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Faculdade de Farmácia

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Neuron Glia Biology in Health and Disease Group

MODULATION OF MICROGLIA REACTIVITY BY S100B IN MULTIPLE SCLEROSIS

Carla Isabel Silveira Ferreira

Dissertação de Mestrado

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS

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Dissertação de Mestrado orientada pela Prof.ª Doutora Adelaide Maria Afonso Fernandes Borralho e pela Doutora Andreia Pereira Barateiro

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À Margarida.

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Abstract

Multiple sclerosis (MS) is a neurodegenerative disease with severe effects on motor and cognitive function. Despite the evolution of knowledge in recent decades, the causes and the exact mechanisms that originate the disease are still unknown. Recent studies demonstrated that S100B protein expression is increased in MS patients and that its high levels are correlated with glial reactivity, contributing to the characteristic excessive inflammatory response of this disease. We also showed that S100B blockade does not prevent demyelination-associated activation of microglia, but it decreases the expression of pro-inflammatory markers. Interestingly, microscopic imaging suggested that upon S100B neutralization microglia moved to myelin surroundings, where they can play an important role on the clearance of myelin debris and remyelination. Thus, we decided to explore whether S100B blockade might modulate the reactivity of microglia in an *ex vivo* demyelinating model.

Therefore, we first evaluated whether S100B might affect demyelination. Our results corroborate a decreased expression of myelin-related protein upon demyelination, which is prevented after S100B neutralization. We also quantified the different populations of reactive microglia showing that there is an augment of M1 pro-inflammatory reactive microglial cells in consequence of demyelination, which is reduced with S100B blockade. Indeed, while upon demyelination, we verified an increase of gene expression of pro-inflammatory mediators, S100B antibody neutralization partially prevented this effect. In turn, we also observed an increase of anti-inflammatory markers usually associated to microglia M2 phenotype, but this time S100B blockade maintained their elevated gene expression. In addition, we verified that S100B neutralization although not increasing the average number of particles phagocytosed by each microglia upon demyelination, it increased the number of microglia with a phagocytic ability. Moreover, also the expression of a neuron-derived microglia calming factor, fractalkine, was enhanced by S100B neutralization suggesting the shift microglia to a damage repair phenotype.

Overall, these results suggest that by neutralizing S100B during a demyelinating event we may prevent the loss of myelin as well as the exacerbation of the inflammatory response, indicating that S100B may be a potential therapeutic target to reduce damage in demyelinating disorders associated with microglial reactivity, such as MS.

Keywords: Multiple Sclerosis; Microglia; S100B; Demyelination

Resumo

A Esclerose Múltipla (EM) é uma doença neurodegenerativa com efeitos graves a nível motor e cognitivo. Apesar da evolução do conhecimento nas últimas décadas, as causas e os mecanismos que desencadeiam a doença são ainda desconhecidos. Estudos recentes demonstraram que a expressão da proteína S100B está aumentada em doentes com EM e que estes níveis elevados estão correlacionados com a reatividade glial, contribuindo para uma resposta inflamatória excessiva, característica desta doença. Verificámos igualmente que o bloqueio de S100B não previne a ativação da microglia aquando de uma situação de desmielinização, mas diminui a expressão de marcadores pro-inflamatórios. Curiosamente, imagens de microscopia sugerem que após a neutralização de S100B a microglia movimenta-se para a zona da mielina, onde pode desempenhar um papel importante na remoção dos detritos de mielina e na remielinização. Assim, decidimos avaliar se o bloqueio de S100B pode modular a reatividade da microglia num modelo *ex vivo* de desmielinização.

Assim, primeiro avaliámos se a proteína S100B pode afetar a desmielinização. Os nossos resultados revelam uma diminuição da expressão génica de uma proteína associada à mielina após desmielinização, a qual é prevenida após a neutralização de S100B. Quantificámos ainda as diferentes populações de microglia reativa, mostrando que há um aumento das células microgliais com um fenótipo M1 pró-inflamatório em consequência da desmielinização, a qual é reduzida com o bloqueio de S100B. De facto, a desmielinização leva a um aumento da expressão génica de mediadores pró-inflamatórios, enquanto a neutralização de S100B previne parcialmente este efeito. Por seu lado, observámos igualmente um aumento dos marcadores anti-inflamatórios associados a um fenótipo microglial M2, mas o bloqueio de S100B manteve a sua elevada expressão génica. Verificámos ainda que a neutralização de S100B apesar de não aumentar o número médio de partículas fagocitadas por cada microglia após desmielinização, elevou o número de células microgliais com capacidade fagocítica. Curiosamente, observámos igualmente que a expressão de um fator derivado dos neurónios que acalma a reatividade microglial, a fractalkina, estava aumentada após neutralização de S100B, sugerindo que a microglia possa ter adquirido um fenótipo mais adequado para a reparação do dano.

De uma maneira geral, estes resultados sugerem que ao neutralizar o S100B podemos prevenir a desmielinização bem como uma resposta inflamatória exacerbada, indicando que a S100B pode ser um potencial alvo terapêutico para reduzir o dano em doenças desmielinizantes associadas à reatividade microglial, tais como a EM.

Palavras-chave: Esclerose Múltipla; Microglia; S100B; Desmielinização

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arg1	Arginase 1
BBB	Blood-brain-barrier
Ca^{2+}	Calcium
CEBP-α	CCAAT-enhancer binding protein α
CD	Cluster of differentiation
CNS	Central nervous system
COSC	Cerebellar organotypic slices culture
CSF	Cerebrospinal fluid
CX3CL1	CX3C chemokine ligand 1
CX3CR1	CX3C chemokine receptor 1
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
EAAT	Excitatory amino acid transporter
EAE	Experimental Autoimmune Encephalomyelitis
Fe^{2+}	Iron
FIZZ-1	Resistin-like alpha ou found in inflammatory zone
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
HMGB1	High-mobility group box 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
K^{+}	Potassium
LPC	Lysophosphatidylcholine or lysolecithin
MBP	Myelin basic protein
MFG-E8	Milk fat globule factor-E8
MHC-II	Major histocompatibility complex, class II
MMP	Metalloproteinase
MS	Multiple sclerosis
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
Na^{+}	Sodium

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
PLP	Proteolipid protein
PPMS	Primary-progressive multiple sclerosis
PRMS	Progressive-relapsing multiple sclerosis
qRealTime PCR	Quantitative real-time polymerase chain reaction
RAGE	Receptor for Advanced Glycation Endproducts
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
SOCS	Suppressor of cytokine signaling
SPMS	Secondary-progressive multiple sclerosis
TGF-β	Transforming growth factor-β
Th	T helper
TNF-α	Tumor necrosis factor-α
TLR	Toll-like receptor

I. Introduction

1. Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disorder of the Central Nervous System (CNS) which leads to the development of focal demyelinated plaques in the white matter (Lassmann, 2011). Furthermore, this disease is characterized by reactivation of antigen-presenting cells, microglial activation, production of cytotoxic mediators and recruitment of systemic immunocompetent cells that leads to a generalized neural tissue damage (Gonsette, 2008).

Generally, MS starts in young adulthood with neuroinflammation, characterized by the CNS infiltration of immune cells across the Blood-Brain-Barrier (BBB), resulting in focal demyelinated plaques formation and axonal damage (Compston and Coles, 2008; Lassmann, 2011; Stadelmann et al., 2011). Depending on plaque location, the symptomatology may be very different (Friese et al., 2014; Lassmann et al., 2012). The immune cell invasion may lead to a permanent activation of macrophages and microglia in parenchyma that result in demyelination and neurodegeneration (Compston and Coles, 2008; Napoli and Neumann, 2010). Therefore, the neurodegeneration which starts with acute lymphocytic inflammation may progress towards chronic inflammation (Ciccarelli et al., 2014).

Demyelination is the total or partial loss of the myelin sheath around axons, thus compromising the efficient conduction of action potentials (Love, 2006). This loss may be a consequence of several factors, including inflammatory processes or viral infections that damage myelin sheaths, which are constituted by two main proteins, proteolipid protein (PLP) and myelin basic protein (MBP); or the cells that synthetize them, oligodendrocytes in the CNS and Schwann Cells in the Peripheral Nervous System (PNS) (Lassmann, 2011; Love, 2006). Axonal myelination is a crucial factor for correct signal transmission and any damage in myelin sheaths may have serious consequences at cognitive and motor levels (Berger and Reindl, 2007;

Lassmann, 2011). Thus, in order to delay disease progression it is important to reduce this demyelination, which is a hallmark of MS, or to promote the remyelination process.

1.1. Clinical course of Multiple Sclerosis

In MS patients usually experience a first neurologic event that is sustained for at least 24 hours. As showed in Figure I. 1, there are 4 types of MS named in accordance with the clinical course of the disease over time: Relapsing-Remitting Multiple Sclerosis (RRMS); Primary-Progressive Multiple Sclerosis (PPMS); Secondary-Progressive Multiple Sclerosis (SPMS) and Progressive-Relapsing Multiple Sclerosis (PRMS).

The RRMS is the most common form of MS. Initially, about 85% of patients are diagnosed with RRMS. This form of MS is characterized by active lesions and temporary relapses that can be partially or completely reversible. The majority of this patients with RRMS progress to a secondary progressive phase (Lindberg and Kappos, 2006), being designated as SPMS, a phase characterized by irreversible deficits and neurodegeneration which are steadily increasing, with or without the occurrence of relapses and remissions (Lindberg and Kappos, 2006).

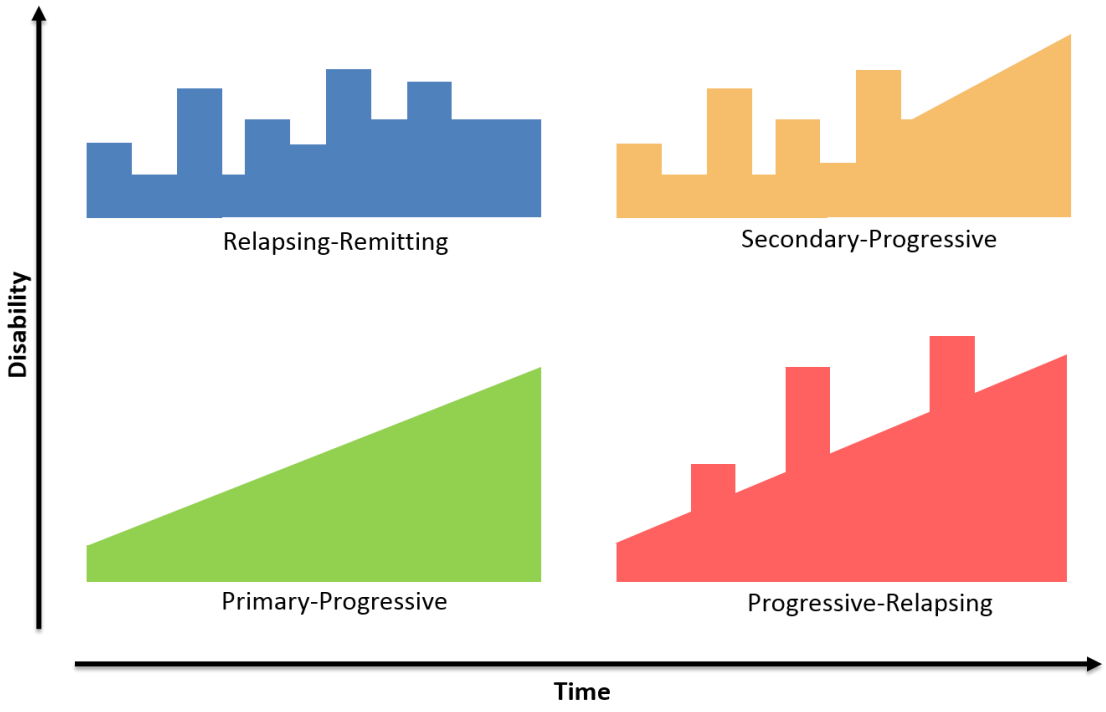

Figure I. 1. Schematic representation of the evolution of disability over time in different types of MS

A small percentage of MS patients (~10%) have PPMS that is characterized by a slow and continuous progression without relapses (Lindberg and Kappos, 2006). The least common form (~5%) is PRMS that is characterized by a steady worsening of neurologic function, with occasional relapses but no complete remissions, with or without recovery. Once PRMS is progressive from onset, it may be diagnosed as PPMS and subsequently changed to PRMS, when a relapse occurs (Lassmann et al., 2012; Milo and Miller, 2014).

In the course of MS it is possible to distinguish different types of focal plaques of demyelination: active, chronic active lesions slowly expanding, inactive lesions and remyelinated shadow plaques. The classic active plaques are characterized by high inflammation with perivascular infiltrates of lymphocytes and macrophages, a complex architecture being commonly detected during the acute or relapsing–remitting stages. The chronic active lesions are considered to comprise approximately 50% of the lesions in progressive stage of disease. The center of this type of lesion usually does not have myelin nor presents remyelination signals, but shows axonal loss. Furthermore, lesions are surrounded by an area of microglial activation and initial tissue injury. The inactive lesions are the most frequent type in all stages of the disease and present signals of an inefficient remyelination, lack of myelin and axonal loss. In addition, the lymphocytic infiltrates and microglial activation are reduced. Finally, the shadow plaques result from remyelination and normally present low axonal injury and thinly remyelinated axons. This remyelination occurs during the acute inflammation, following myelin debris removal by phagocytosis, but it may also be detected in the progressive phase (Lassmann, 2010; Lassmann et al., 2012; Love, 2006).

Once the disease has an early and strong inflammatory component, treatments targeting the inflammatory insult have been shown to be effective mostly in the relapsing stage. However, in the progressive phase of disease, the anti-inflammatory or immunomodulatory treatments used so far showed no clinical relevance (Lassmann, 2011). Thus, a key step for the treatment of progressive MS is the development of new therapies for inflammatory and neurodegenerative components of the disease.

1.2. The etiology of MS

The etiology of MS is not fully understood yet, but in the majority of patients, the disease progression is characterized by acute relapses (RRMS) leading to a progressive and irreversible accumulation of neurological deficits (SPMS). Relapses are the manifestations of inflammatory demyelinating lesions in the CNS (Charil and Filippi, 2007; Compston and Coles, 2008; Love,

2006). In a primordial phase, the inflammation is a transitory feature and is followed by remyelination that contributes to total or partial clinical remission of the symptomatology. However, this remyelination may not be complete and, over time, repeated acute insults and the failure of reparative system may lead to extensive microglial activation associated with irreversible axonal and neuronal loss resulting into neurodegeneration (Charil and Filippi, 2007).

For many years, the focus of MS research had been on inflammatory white matter pathology, once the disease was initially considered to be an immune-mediated demyelinating disorder. However, there are results that show axonal loss occurring in early phase of disease (Lindberg and Kappos, 2006). These evidences suggest a strong neurodegenerative component, which contributes to pathogenesis and reveal that MS may be an inflammatory demyelinating and neurodegenerative disease that affects all the CNS (Herz et al., 2010). One hallmark of MS are in fact demyelinated lesions in white matter related with axonal degeneration and immune cells infiltration. The focal demyelinated plaques, present in the grey and white matter at all stages of the disease, are infiltrated by populations of immune cells and immune mediators such as T cells, B cells, macrophages and microglia, as well as cytokines, chemokines and other toxic agents (Lassmann et al., 2012; Napoli and Neumann, 2010). The infiltration of immune cells is a consequence of BBB disruption, partly due to matrix metalloproteinases (MMPs). The expression of several MMPs, particularly MMP-2 and -9, are also altered in microglial cells of MS lesions (Könnecke and Bechmann, 2013; Rosenberg, 1995).

Besides demyelination, immune cell-related inflammation is critical for neuronal damage due to pro-inflammatory neurotoxic substances release and consequent damaging processes. In these patients, the inflammatory lesions consist of perivascular and parenchymal infiltrates of lymphocytes and macrophages (Lassmann et al., 2012). While in active lesions, there are low levels of T cells at sites of the initial tissue injury, the ongoing tissue damage is associated with the infiltration of macrophages and the activation of resident microglia. In addition, the brains of these patients also display global changes as widespread inflammation, microglial activation, astrocytic gliosis and slight demyelination and axonal loss in normal-appearing white matter. These changes together with widespread loss of tissue volume observed in the cortex result in brain atrophy with ventricles dilatation (Herz et al., 2010; Lassmann et al., 2012).

1.3. Molecular mechanisms of neurodegeneration in Multiple Sclerosis

As a result of inflammatory processes many molecular changes occur in CNS during MS, namely the secretion of neurotoxins that induce immune responses with important roles in homeostasis and neuronal metabolism. These responses have different functions depending on the time of exposure. At short-term, the immune response has a crucial role in tissue defense but, at long-term, immune cells induce stress responses (Friese et al., 2014). The molecular pathways involved in MS neurodegeneration are very complex due to the heterogeneity of this disease. Although most of these mechanisms are also associated with other neurodegenerative diseases, the extensive primary demyelination and preservation of axons is specific for MS. It is therefore necessary to understand the pathogenesis of MS and identify the mechanism that specifically affects myelin and the cells responsible for its production, contributing to widespread primary demyelination (Lassmann, 2013). After this first myelin destruction due to inflammation, other known mechanisms may be responsible for axonal loss, worsening MS clinical course.

1.3.1. Free Radicals and Oxidative Stress

Oxidative stress reflects an imbalance between the reactive oxygen species (ROS) production and the ability to detoxify this reactive species and repair the damage (Mao and Reddy, 2010). Although it cannot be generalized, there are evidences that in some patients with MS, oxidative stress may be the principal mechanism implicated in the pathogenesis of disease (van Horssen et al., 2008).

Oxidative stress is able to damage the cells by promoting the oxidation of cellular components, as lipids, proteins and nucleic acids (mainly mitochondrial DNA, mtDNA), which consequently leads to cell death (Mao and Reddy, 2010). Specifically, it was verified an accumulation of oxidized DNA and lipids within lesions in all stages of the disease. However, in active lesions there is a greater indication of oxidative damage than in inactive lesions (predominate in the progressive stage of MS), which presents a low signal (Haider et al., 2011).

As already described, the excessive inflammatory environment in demyelinating lesions is favorable to ROS and reactive nitrogen species (RNS) formation, and this increased levels of reactive species may compromise the antioxidant defenses in our organism, particularly in the lesions (Mao and Reddy, 2010). In support of these data, it was observed an increased expression of enzymes responsible for free radical production (e.g. myeloperoxidase, nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase) in active lesions,

mainly in areas of initial tissue injury (Fischer et al., 2012). The presence of superoxide and peroxynitrite, which are produced due to ROS and RNS reaction, as well as the increased levels of DNA oxidation within plaques shows that the generation of reactive species may have an extremely toxic effect in neuronal and glial cells (Mao and Reddy, 2010). In support of these evidences, the presence of oxidized DNA and lipids in apoptotic oligodendrocytes and dystrophic axons also indicates the key role of ROS in demyelination and neurodegeneration (Lassmann et al., 2012).

Despite the fact that anti-oxidative activity is not different in MS patients, comparatively with healthy controls, there are evidences that sulfhydryl groups, which have antioxidants properties, are decreased in MS patients (Mao and Reddy, 2010). However, some antioxidant enzymes (e.g. superoxide dismutases, catalase, peroxiredoxins) are upregulated in active MS lesions, which may indicate an active defense mechanism to reduce cellular damage caused by ROS (van Horssen et al., 2008).

Oligodendrocytes are particularly sensitive to higher levels of these reactive species, comparatively with astrocytes and microglia. This fact may result from their reduced ability for antioxidant defense, rendering them more prone to oxidative stress toxicity which leads to oligodendrocyte death and consequent demyelination. Furthermore, ROS and RNS can also damage the own myelin sheath and promote its clearance by macrophages and microglia. In an early stage of MS, the oxidative stress appears to be triggered by activated microglia but, in a progressive stage, it may be intensified by further factors (Friese et al., 2014; Lassmann, 2013; Mao and Reddy, 2010).

1.3.2. Mitochondrial dysfunction

Mitochondria has as principal function to provide energy to cells in the form of ATP. It participates in many cellular processes, including fatty acid oxidation, apoptosis and calcium homeostasis. So, the high energy needed by CNS render it slightly vulnerable to mitochondrial damage.

A serious consequence of high levels of ROS is mitochondrial dysfunction, which may result from different mechanisms. The released free radicals and modifying proteins can disrupt mitochondrial function by interfering with some components of respiratory chain and promoting mtDNA damage (Ellwardt and Zipp, 2014; Lassmann et al., 2012). These mitochondrial changes may explain pathological features of MS lesions as demyelination and remyelination impairment, destruction of thin-calibre axons, differentiation arrest of

oligodendrocyte progenitor cells, oligodendrocyte apoptosis and astrocyte dysfunction (Haider et al., 2011; Lassmann et al., 2012).

Mitochondria plays several crucial functions in different pathways including oxidative energy metabolism, where the most of the ATP is synthesized. Therefore, it is easy to understand that the impairment of mitochondria, besides leading to the production of more reactive species that will exacerbate the tissue injury (Mao and Reddy, 2010), will also induce energy failure. Indeed, it is clear that mitochondrial injury and consequent energy failure is a very important factor that drives to MS tissue injury (Lu et al., 2000; Witte et al., 2010).

The first evidence that the mitochondrial damage has some role in MS lesions demonstrated a compromised NADH dehydrogenase activity as well as an increase of complex IV activity within lesions (Lu et al., 2000). Active lesions show significant changes in proteins of the mitochondrial respiratory chain and, in addition, deletions in mitochondrial DNA are present in neurons, especially in the progressive stage of the disease.

Concerning oligodendrocytes, the mitochondrial damage results on release of apoptosis-inducing factor (AIF), which translocates into the nucleus and induces DNA damage. With the damage, one polymerase (poly ADP-ribose polymerase) is activated in an attempt to repair the injury, however, this leads to further energy deficiency (Lassmann et al., 2012).

There are also suggestions that activated microglia play an important function in mitochondrial dysfunction, namely in MS. Activated microglia are responsible for ROS and NO production that, besides damaging mtDNA, can lead to the inhibition of oxidative phosphorylation pathway disrupting the ATP production and increasing ROS formation (Witte et al., 2010, 2014). Additionally, there has been reported a reduction in PGC-1α (peroxisome proliferator-activates receptor gamma coactivator-1α), a transcriptional co-activator and regulator of mitochondrial function, in MS cortex. This decreased in PGC-1α levels coinciding with both reduced expression of subunits involved in oxidative phosphorylation pathway and decreased expression of several mitochondrial antioxidants (Witte et al., 2013). Therefore, mitochondria has a decreased capacity to produce ATP and detoxify oxidative stress, which can compromise its efficient functioning.

1.3.3. Ion channel dysfunction

The intracellular environment is very important for maintaining neuronal functions. Abnormal expression of Na^+ channels, acid-sensing Na+ channels, glutamate receptors and voltage-gated Ca^{2+} channels has been detected in dystrophic and demyelinated axons. In this

context, the ion channel dysfunction has a great impact on neurons and axons, which might even lead to their degeneration and death in progressive phase of MS (Friese et al., 2014; Lassmann et al., 2012; Mao and Reddy, 2010). As already mentioned, in response to an inflammatory stimulus, energy imbalance and demyelination may lead to activation, dysfunction and altered distribution of ion channels, inducing downstream mechanisms. These mechanisms are mostly responsible for Ca^{2+} accumulation and apparently may be the promoters of neurotoxicity and trigger of innumerous enzymes activation, which compromise both the normal mitochondrial functioning and axonal transport and result in additional increase of Ca^{2+}levels. Moreover, the wrong distribution of voltage-gated Ca^{2+} channels (VGCCs) in demyelinated fibers may lead to an abnormal influx of Ca^{2+}, which contributes to axonal death (Friese et al., 2014).

The Na^+ channels are responsible for the acceleration of the saltatory conduction in myelinated axons, which is the propagation of an action potential from one node of Ranvier to another, along a myelinated fiber. When into the axon, Na^+ is exchanged for K^+ by Na^+/K^+-ATPase, and this ion exchange is important for axonal polarization. This enzyme is responsible for correcting Na^+ and K^+ levels and for preventing a pathological influx of Na^+ in axons, however, in pathological conditions, inflammatory mediators are released and cause the Na^+/K^+-ATPase failure and consequent mitochondrial injury (Mao and Reddy, 2010). It has also been shown that under hypoxia, this Na^+/K^+ pump activity are inhibited and that reactive species increase its degradation (Waxman, 2008; Young et al., 2008).

1.3.4. Excitotoxicity of Glutamate

Glutamate has been found in MS lesions at high concentrations (Srinivasan et al., 2005). It is one of the most important excitatory neurotransmitter of the CNS and modulates ion homeostasis into the cells through several receptors including N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors. The glutamate-mediated excitotoxicity is an important connection between neuroinflammation and neurodegeneration, once high levels of glutamate stimulate its receptor and lead to deregulation in ion homeostasis contributing to neurotoxicity associated with axonal, oligodendroglial and myelin damage or even cell death (Ellwardt and Zipp, 2014; Takaki et al., 2012). So, excessive levels of glutamate, which are released by activated immune cells including activated microglia, may contribute to the lesion development in MS by overstimulation of ionotropic receptors (Pitt et al., 2000). This involvement in lesion development is supported by results that demonstrate a reduction of neurological disability and axonal damage, and an

increase of oligodendrocyte survival with AMPA/kainate receptor antagonist treatment. Thus, these results show that AMPA/kainate-mediated glutamate excitotoxicity has an important role in CNS damage in the animal model of MS, the experimental autoimmune encephalomyelitis (EAE) and probably also in the course of MS (Pitt et al., 2000).

Also the activation of microglia seem to be involved in the impairment of glutamate transporters. Domercq and collaborators showed that high ROS levels released by activated microglia inhibit glutamate uptake by oligodendrocytes, resulting in extracellular glutamate increase (Domercq et al., 2007). In addition, it has further been demonstrated that activated microglia are correlated with focal loss of excitatory amino acid transporters, EAAT1 and EAAT2. They also verified alterations in the mechanisms of glutamate uptake only in the presence of activated microglia (Vercellino et al., 2007). Once EAATs are fundamental in both the maintenance of low extracellular glutamate levels and in prevention of excitotoxicity as well, activated microglia appear to have an important role in excitotoxicity present in MS.

1.3.5. Iron accumulation

It is known that iron accumulates in healthy human brain with age increase. This fact may be relevant once progressive phase of MS usually starts between 40 and 50 years of age (Lassmann et al., 2012).

Iron, which is mainly stored in oligodendrocytes, is crucial for normal brain metabolism, including for myelination, however, it may generate ROS (Friese et al., 2014; Hametner et al., 2013). As consequence of oxidative stress, activated microglia release H_2O_2 that diffuses into oligodendrocytes with Fe^{2+} accumulation and forms toxic radicals leading to cell death. The Fe^{2+} released from these dying oligodendrocytes is taken up by microglial cells, which subsequently become dystrophic. These dystrophic microglia release more Fe^{2+} that results in more oxidative tissue injury. So, iron accumulation within oligodendrocytes is also a relevant mechanism that further contribute to neurodegeneration in MS (Ciccarelli et al., 2014; Lassmann et al., 2012).

1.3.6. Inflammatory mediators

Myelin sheaths loss occurs due to the migration of auto-reactive immune cells through the BBB that attack myelin components. Within the CNS, activated T-cells together with activated microglia, macrophages and astrocytes, release pro-inflammatory cytokines, creating a pro-inflammatory environment which can lead to neurodegeneration. The accumulation of

these pro-inflammatory mediators, which are described in section 1.4.1.2, amplify both inflammatory and immune response contributing to demyelination and then neurodegeneration (Dendrou et al., 2015; Glass et al., 2010; Lassmann and van Horssen, 2011; Vogel et al., 2013). Besides all these inflammatory contributors, including high levels of S100B, the protein in which we are interested, stimulate the release of further inflammatory mediators, meaning that it has a function on exacerbating the inflammatory response (Bianchi et al., 2010; Villarreal et al., 2014).

1.4. Microglia as cellular players in Multiple Sclerosis

As mentioned above, in all phases of MS, active tissue injury is associated with inflammatory infiltrates. In addition, several immune cells including activated microglia are observed in lesions borders. These cells express cytokines and enzymes involved in the production of ROS and RNS (Fischer et al., 2012), having a crucial effect on MS pathogenesis.

1.4.1. Microglial cells

Microglia are the tissue macrophages of the brain and the main form of immune defense in the CNS constituting around 10% of the cells in this system. They are members of the innate immune system and respond to danger signals, initiating an acute inflammatory response, within the CNS (Goldmann and Prinz, 2013; Jack et al., 2005). These cells have functions similar to those of other tissue macrophages such as phagocytosis, antigen presentation and secretion of cytokines (Herz et al., 2010). Microglia act quickly after an insult in order to restrain the damage and promote recovery. However, besides promoting neuroprotection and stimulating tissue repair, activated microglial cells can exacerbate an inflammatory status and trigger neurotoxic pathways, which may lead to a progressive neurodegeneration (Correale, 2014).

An important characteristic of microglia are their extensive branches, which allows that these cells continuously patrol the CNS parenchyma (Benarroch, 2013; Olah et al., 2011a). This characteristic makes microglial cells the first line of defense in CNS (Correale, 2014; Giunti et al., 2014; Olah et al., 2011a). As consequence of brain injury, resident microglia change their surveillance phenotype to an “activated” morphology, which are associated with different phenotypes highly dependent on the type, intensity and duration of their exposure to stimuli (Benarroch, 2013; Correale, 2014; Perry et al., 2010). These different microglial phenotypes may

be defined based on morphological, molecular and functional characteristics (Kettenmann et al., 2011).

1.4.1.1. Surveillant microglia

Under healthy conditions, microglial cells display a surveillant/patrol or M0 phenotype, which are characterized by a ramified morphology, a slow turnover rate and low expression of surface molecules. This apparently "quiescent" microglia are constantly scanning their environment for exogenous or endogenous signals (Giunti et al., 2014; Kettenmann et al., 2011), and are ready to rapidly switch to the "activated" state, after injury occurrence (Kettenmann et al., 2011).

Surveillant microglia phenotype seems to be preserved through interactions between some receptors and their respective ligands expressed in neurons, such as CD200-CD200R and fractalkine (CX3CL1)-CX3CR1, which are described as "off signals" (Correale, 2014; Jones and Lynch, 2014; Perry et al., 2010). A study that reveals the presence of a microglia mainly activated in CD200 knockout mice comparatively with control mice, clearly demonstrated the importance of these interactions in the surveillant phenotype (Hoek, 2000). Besides this, neurons are also responsible for several neurotrophic factors release (e.g. nerve growth factor and brain-derived neurotrophic factor) that keep the microglial cells at rest (Perry and Teeling, 2013).

1.4.1.2. Activated microglia

Any disturbance, which may indicate a potential danger to the CNS changes microglial morphology, gene expression and their functional behavior (Correale, 2014; Kettenmann et al., 2011). Through their branches, microglia sense variations in their microenvironment, recognize danger signs and consequently become activated. Morphologically, microglia retract their branches changing their shape to an amoeboid form (Correale, 2014). Moreover, microglia become motile and go to the lesion following chemotactic gradients. In addition, CNS injury induces microglia proliferation that provide more cells for protection and repair of tissue homeostasis (Benarroch, 2013; Kettenmann et al., 2011).

Besides all these alterations, microglia display molecular alterations such as upregulation of some surface markers and specific proteins, presenting a classically activated (M1) or an alternative activated (M2) phenotype (Chhor et al., 2013; Correale, 2014; Giunti et al., 2014; Goldmann and Prinz, 2013). M1 consists of a pro-inflammatory phenotype mainly

associated with cytotoxic response, which is responsible for mediating innate immune responses but also adaptive immune responses. The innate immune response may be triggered by damage-associated molecular patterns (DAMPs), misfolded proteins and other proteins released from injured neurons, which in turn activates toll-like receptors (TLR), in microglial cells. In response to this interaction, TLRs activate downstream signaling cascades leading to transcriptional activation of nuclear factor kappa-B (NF-κB) and subsequent acute inflammation. This feature is characterized by the production ROS, that leads to neuronal death, engagement of NLRP3 inflammasome, expression of altered enzymes and surface markers and release of pro-inflammatory cytokines, as shown in Table I. 1 (Benarroch, 2013; Chhor et al., 2013; Correale, 2014; Goldmann and Prinz, 2013). The activation of inflammasome by DAMPs enhances the inflammatory response. Also the high-mobility group box 1 (HMGB1), a protein that is secreted by damage neurons for signaling the cell damage, can interact with TLRs, among others, further activating microglia and exacerbating the NF-κB signaling cascade (Brites and Vaz, 2014). Additionally, the adaptive immune response is triggered by interferon gamma, which is released from T helper cells type 1 (Th1), and in response to that, microglia become antigen-presenting cells and release pro-inflammatory mediators as effectors of adaptive immunity (Benarroch, 2013).

Table I. 1. Characteristics of different microglial phenotypes (compiled from Brites and Vaz, 2014; Correale, 2014; David and Kroner, 2011; Liu et al., 2008)

Phenotype	Inflammatory mediators released	Phenotypic markers
Classically activated microglia, M1	- Tumor necrosis factor alpha (TNF-α) - Interleukin (IL)-1β - IL-6 - Milk fat globule factor-E8 (MFG-E8)	- Inducible nitric oxide synthase (iNOS) - Cluster of differentiation 45 (CD45) - CD86 - Major histocompatibility complex, class II (MHC-II)
Alternative activated microglia, M2a	- IL-10 - Transforming growth factor-beta (TGF-β)	- Arginase 1 (Arg1) - Resistin-like molecule alpha or protein found in inflammatory zone (FIZZ-1) - Suppressor of cytokine signaling 1 (SOCS-1)
Immunoregulatory, M2b	- IL-10 - TNF-α - IL-1β - IL-6	- SOCS-3
Acquired-deactivating, M2c	- IL-10 - TGF-β - MFG-E8	- Arg1 - SOCS-3

M2 microglia phenotype plays a crucial role in repair and healing of tissues, once microglia secrete extracellular matrix proteins and growth factors, and promote phagocytosis of cellular and myelin debris contributing for the remyelination process. Moreover, these microglia are also known for their involvement in synapse repair and remodeling (Correale, 2014). M2 microglial phenotype is induced by signals from apoptotic cells, as heat shock protein 60 (Hsp60), that activates triggering receptor in myeloid cells-2 (TREM-2), or by anti-inflammatory cytokines such as interleukin (IL)- 4 and IL-13, which are released from Th2 helper cells (Benarroch, 2013; Olah et al., 2011b). Thus, this phenotype seems to be beneficial compared with M1 however, a prolonged activation of this M2 phenotype may be harmful for preventing axonal growth (Brites and Vaz, 2014). In addition, there are three different subclasses of M2 microglia, M2a, M2b and M2c, which are induced through polarizing signals and have different functional properties. M2a is an alternative activation repair/remodeling phenotype, which is recruited for phagocytosis and inflammation repair and induced by IL-4 and IL-13, and express specific markers, as presented in Table I. 1 (Brites and Vaz, 2014; Chhor et al., 2013; Varnum and Ikezu, 2012). Moreover, M2a microglia release anti-inflammatory mediators that trigger an anti-inflammatory response and promote tissue repair (Correale, 2014; Goldmann and Prinz, 2013). The immunoregulatory or M2b phenotype is stimulated through immune complexes, TLR agonists and IL-1R ligands. Although M2b microglia release IL-10, an anti-inflammatory cytokine, they also release some pro-inflammatory cytokines (Table I. 1), which suggests that M2b microglial cells may act in and modulate different conditions of inflammation (Chhor et al., 2013; David and Kroner, 2011). The specific markers of M2b phenotype are SOCS-3 and IL-1R antagonist (IL-1Ra) (Chhor et al., 2013). The acquired-deactivating or M2c phenotype is induced by IL-10, TGF-β, glucocorticoids and enhances anti-inflammatory marker expression (Table I. 1) while decreasing pro-inflammatory cytokine levels (Chhor et al., 2013; Varnum and Ikezu, 2012). Besides modulating the anti-inflammatory polarization through the downregulation of pro-inflammatory markers, SOCS-1 also regulates M2 phenotype, since the expression of SOCS-1 is increased in M2 phenotype (Davey et al., 2006; Guedes et al., 2013; Wilson, 2014).

As already mentioned, an important neuroprotective function of microglial cells is phagocytosis. M1 and M2c microglia can produce MFG-E8 that recognizes the phosphatidylserine (PS) exteriorized by apoptotic cells and triggers a signaling cascade that stimulates the phagocytic process of dying cells (Brites and Vaz, 2014).

1.4.1.3. Microglia in MS

The role of microglia in MS is controversial. Several evidences indicate that classically activated microglia promotes neuroinflammation and demyelination in MS and in EAE, contributing to CNS injury. To elucidate the function of microglial cells, it has been shown that the inhibition of microglial activation results in a delayed EAE onset (Bogie et al., 2014; Giunti et al., 2014; Heppner et al., 2005). In addition, microglial activation has been observed in *post-mortem* brain tissue of MS patients as well as the presence of clusters of activated microglia within the normal-appearing white matter (Giunti et al., 2014; van Horssen et al., 2012). Our group also observed a large population of activated microglia/macrophages and PLP-positive macrophages in active MS lesions in contrast to chronic MS lesions (Barateiro et al., 2015) (Figure I. 2).

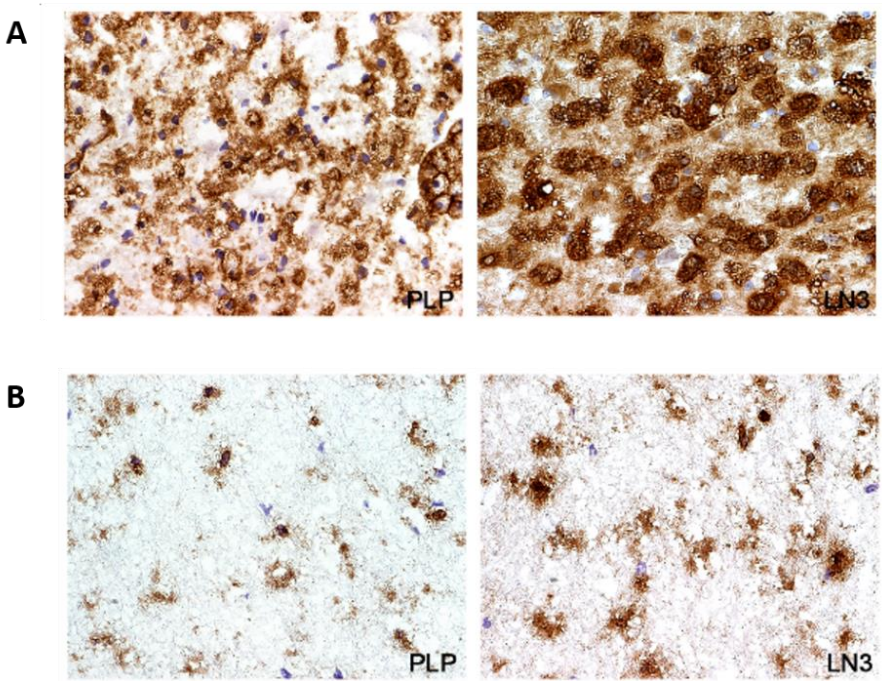

Figure I. 2. Presence of microglia in active (A) or chronic (B) MS lesions. Frozen sections of autopsied brain samples of MS patients were immunostained for PLP to detect white matter and for HLA-DR MHC class II clone LN3 to detect macrophages/microglial cells. **(A)** Active MS lesions were outlined by PLP staining and LN3 immunoreactivity. Magnification ×43. In contrast, **(B)** chronic MS lesions showed decreased PLP and LN3 immunostaining. Magnification ×43. (Adapted from Barateiro et al., 2015).

However, all activation states of microglia may be present in disease indicating that there are multiple populations of microglia occurring in the course of a demyelinating episode (Goldmann and Prinz, 2013). Thus, microglia can also present an alternative activated phenotype in EAE or MS, with the ability to phagocyte apoptotic cells and myelin debris, a crucial step for tissue regeneration. In this context, the role of TREM-2, a phagocytic stimulator, was evaluated

and it has been demonstrated that this receptor is upregulated in EAE, facilitating the debris clearance. Moreover, its blockade results into EAE exacerbation with more infiltrates and demyelination in the parenchyma (Piccio et al., 2007).

A study that evaluated the role of microglia/macrophages polarization in remyelination showed that a switch from an M1- to an M2-phenotype is essential for the remyelination process. Particularly, they observed that this change occurs at the time of oligodendrocytes differentiation from oligodendrocyte progenitor cells, which have been recruited into the lesion. Thus, they demonstrated that M2 microglia are necessary for oligodendrocyte differentiation and maturation, which are very important for the efficient remyelination (Miron et al., 2013).

Given that microglial cells present distinct roles along disease progression, it is important to understand how they exert a beneficial role in MS with the objective to modulate this reactivity to a more neuroprotective phenotype during the course of the disease.

2. S100B

S100 proteins are part of a low-molecular-weight and acidic proteins family that are known to contain two distinct EF-hand helix-loop-helix calcium-binding sites (Donato et al., 2013). This family is subdivided into three subgroups according to their action: (i) only exert intracellular regulatory effects, (ii) have intracellular and extracellular functions, or (iii) play extracellular regulatory effects (Donato et al., 2013). S100B is a 10.5 kDa member of this family, with both intracellular and extracellular functions, that is expressed in some cell types from different tissues and that appears to be expressed at highest levels, in the CNS, namely by glial cells (Adami et al., 2001; Michetti et al., 2012). Usually, this protein exists within cells as a homodimer but it can also form heterocomplexes when associated with S100A1 monomer (Donato et al., 2009).

Due to important functions that S100B presents in proliferation, migration, differentiation and apoptosis, it plays a critical role during brain development acting as a neurotrophic factor, by furthering neurite outgrowth and neuronal survival for low/physiological levels (nM) (Donato et al., 2013; Koppal et al., 2001).

On the other hand, it was also demonstrated that S100B expression is increased in many tumors as well as in the aging brain and in the brain of patients affected by different pathologies as Alzheimer's disease, HIV infection, chronic epilepsy and other brain pathological conditions as MS (Donato et al., 2009; Hein Née Maier et al., 2008). Once these high concentrations of

S100B are present in several brain pathologies, this protein may be considered a biomarker of brain damage (Donato et al., 2013).

2.1. Dual role of S100B in physiology and pathology

As mentioned above, S100B is a Ca^{2+} sensor, so, upon binding to Ca^{2+}, S100B changes its conformation leading to hydrophobic patch exposure to the solvent and its interaction with target proteins (Sorci et al., 2013). Several of these target proteins have been already identified and its interaction with S100B is involved in important intracellular processes (Table I. 2). Accordingly, S100B acts as a stimulator of proliferation and migration as well as an inhibitor of apoptosis and differentiation. Moreover, S100B is involved in the regulation of energy metabolism, transcription, and protein phosphorylation, as well as in Ca^{2+} homeostasis (Donato et al., 2009; Sorci et al., 2010).

It is known that astrocytes are the main cell type in CNS that express and secrete S100B, however, other S100B-expressing cells can release the protein in case of damage or necrosis (Sorci et al., 2010). Therefore, besides having a regulatory function within the cytoplasm where it is expressed, S100B can act as a signal molecule in extracellular space given that it can be released by damaged cells (Rustandi et al., 2000; Shashoua et al., 1984; Sorci et al., 2010). Once released, S100B may have a beneficial or harmful action. There are evidences that this release may be mostly dependent on the presence and activation of its receptor RAGE (Receptor for Advanced Glycation Endproducts), which engagement may stimulate further S100B release (Donato et al., 2009; Sorci et al., 2013).

RAGE is a member of the immunoglobulin-like cell surface receptor superfamily composed by a cytosolic domain responsible for signal transduction, a transmembrane domain which anchors it in the membrane, a variable binding-domain and two constant domains (Ostendorp et al., 2007; Sparvero et al., 2009). This receptor is able to transduce inflammatory stimuli and the effects of neurotrophic and neurotoxic factors and therefore, S100B emerged as a damage-associated protein that regulate inflammation-related events and play a role in pathophysiology of neurodegenerative disorders and inflammatory brain diseases (Bianchi et al., 2011; Donato et al., 2013; Zhang et al., 2011b).

Table I. 2. S100B target proteins involved in intracellular processes (adapted from Donato et al., 2013)

Intracellular Function	Target Protein
Ca^{2+} homeostasis	- AHNAK - Annexin 6
Cell locomotion	- Src/PI3-K/RhoA/ROCK - Src/PI3-K/Akt/GSK3β/Rac1
Cell proliferation and differentiation	- p53 - PI3-K/Akt/$p21^{WAF1}$/cdk4/Rb/E2F - IKKβ/NF-κB
Channel Activity	- EAG1 K^+
Cytoskeleton	- Microtubules - Type III intermediate filaments - Caldesmon - Calponin - IQGAP1
Enzymes	- Membrane-bound GC - Fructose-1,6-biphosphate aldolase - Phosphoglucomutase - Twichin - Ndr - Src
Protein degradation	- E3 ligase hdm2
Protein phosphorylation	- p53 - τ proteins - GAP43 - p80 - GFAP - Vimentin
Receptor function	- D2 receptor/$ERK_{1/2}$/adenyl cyclase
Transcription factors	- p53

Besides having different functional roles, the extracellular form of S100B, which has more effects in CNS, presents different effects on neurons, astrocytes and microglia, depending on the concentration (Donato et al., 2013). At physiological levels the protein displays trophic effects on neurons promoting neuron survival and growth as well as microglia quiescence, although high concentration of S100B display pro-inflammatory effects and activate pro-apoptotic pathways (Reali et al., 2005). In particular, high levels of S100B can stimulate the nitric oxide synthesis by astrocytes and microglia leading to neuronal and astrocyte apoptosis. Moreover, high S100B stimulates the release of cytokines contributing to brain inflammatory response (Bianchi et al., 2011).

2.1.1. Extracellular S100B effect on microglial cells

The high expression levels of S100B are normally associated to astrogliosis in the course of neurodegenerative diseases. These levels might be the result of neuronal and glial cells pathology given that the protein is released by damaged oligodendroglial or astroglial cells, which have will then activate microglial cells responsible for the innate immune response (Sorci et al., 2010). However, expression levels of S100B exerts two different roles in microglia depending on concentration.

At physiological concentrations, S100B can prevent microglia activation via STAT3 pathway and may also act as a signaling trophic protein that promotes a more protective phenotype of microglia (Zhang et al., 2011b). On the other hand, high S100B concentrations, in the presence of bacterial endotoxin or interferon-γ (IFN-γ), play a main role on microglia activation, exacerbating brain inflammatory response. This activation was shown to be mediated by stimulation of iNOS leading to an increase of nitric oxide release (Adami et al., 2001; Bianchi et al., 2007).

In addition, there is an evidence that S100B stimulates the microglia migration via RAGE-dependent mechanism. It was demonstrated that once S100B accumulates in the extracellular space after brain damage, the increased S100B levels might contribute to intensify the inflammatory response by activating microglia and stimulating their migration (Bianchi et al., 2011).

2.2. S100B in MS

Increased S100B levels were first detected in cerebrospinal fluid (CSF) of MS patients (Michetti et al., 1979). Another study revealed that S100B levels in CSF were higher in patients with RRMS than in patients with SPMS (Bartosik-Psujek et al., 2011). We confirmed that both in CSF and in serum samples from RRMS patients there is a significant increase of S100B production at the time of diagnosis (Barateiro et al., 2015). S100B increase may exacerbate the release of inflammatory mediators and subsequently contribute to neuronal death (Hu et al., 1996). These results indicate S100B as a potential biomarker for MS diagnosis and prognosis, possibly helping to distinguish between relapsing remitting and progressive phases of MS.

One study reported the presence of S100B in acute lesions of *post-mortem* brain tissue of patients with RRMS (Petzold et al., 2002). In similar samples, our recent work demonstrated that S100B is overexpressed both in active and chronic lesions, mainly by astrocytes (Figure I. 3).

In addition, it showed that in chronic lesions S100B is still diffusely expressed into the demyelinated area (Barateiro et al., 2015). Interestingly, those active MS lesions also showed an increase of RAGE expression that co-localized with microglia/macrophages cells, while almost no RAGE was found in chronic lesions that are almost depleted of microglia/macrophages (Figure I. 3).

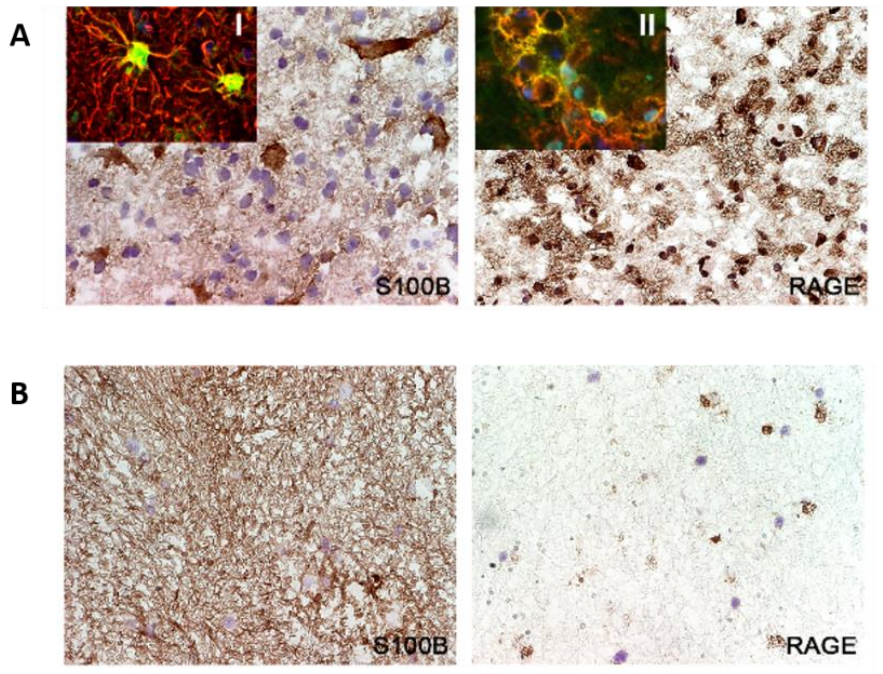

Figure I. 3. Expression of S100B and its receptor RAGE in active (A) and chronic (B) MS lesions. Frozen sections of autopsied brain samples of MS patients were immunostained for S100B and RAGE. **(A)** S100B and its receptor RAGE are markedly expressed in active MS lesions by astrocytes and activated macrophages/microglia, respectively. Magnification x40. Insets show the co-localization of (I) glial fibrillary acidic protein (GFAP, red), an astrocytic marker, with S100B (green) and the co-localization of (II) LN3 (red), a marker of activated macrophages/microglia with RAGE (green). Magnification x63. **(B)** S100B but not RAGE is continuously expressed in chronic MS lesions. Magnification x40. (Adapted from Barateiro et al., 2015).

Given the increased expression of S100B during MS episodes, the role of S100B in different neurodegenerative diseases and its apparent involvement in microglia activation, it is of utmost importance to assess whether by modulation of S100B function it is possible to change the reactivity of microglia to a more neuroprotective phenotype during the course of MS.

3. Experimental models to study MS pathophysiology

Failure to understand the neurodegenerative mechanisms involved in MS and therapeutic inefficacy of diverse treatments led to development of multiple experimental

models that mimic the hallmarks of the disease. However, the complexity of MS still hinders the development of the perfect model for this disease (Murta and Ferrari, 2013).

The EAE, a model characterized by inflammation, demyelination and neurodegeneration, is one of the most widely used *in vivo* model to study MS that involves the immunization of genetically susceptible animals with a myelin protein inducing brain inflammation and destruction of myelin. However, EAE models fail to expect the clinical efficacy in patients (Mathew et al., 2013; Ransohoff, 2012). Moreover, *in vivo* models entail an expensive cost, as well as ethical problems and, therefore, alternative models should be used whenever possible.

For decades, organotypic slice cultures were used in CNS research due to three-dimensional architecture, maintenance of contact between different cells and the presence of all the cells of CNS which play a key role in lesion recovery (Denic et al., 2011; Gähwiler, 1984). Organotypic slice culture is a more complex model to study cell-cell interactions, being named *ex vivo*, and can be prepared from different brain regions, such as cerebellum, hippocampus, striatum, cortex and spinal cord, (Birgbauer et al., 2004; Gähwiler, 1984; Stoppini et al., 1991). The preferential region used in MS research, compared with other CNS regions is cerebellum due to the abundance of white matter and the well-known pattern of myelin tracts (Zhang et al., 2011a).

This method was initially used for electrophysiological studies, in 1941, but the process of myelination was only reported in 1956, in cerebellar slices (Hild, 1956; Levi and Meyer, 1941). In 2004, lysophosphatidylcholine (LPC) was used to demyelinate rat cerebellar slices, which was followed by the reappearance of myelin sheaths, suggesting remyelination (Birgbauer et al., 2004). Thus, LPC can be used as a good molecule to induce demyelination in cerebellar organotypic slice cultures (COSC), providing a model that allows the study of demyelination and remyelination in an *ex vivo* model. This model offers advantages compared with other *in vitro* models, once it can mimic the multicellular complexity as well as the structure and functionality of *in vivo* conditions (Cho et al., 2007). Besides preserving glial cells contribution into myelination-associated processes, organotypic slice model excludes the systemic immune system interactions that would render the model more complex (Miron et al., 2010). Thus, this *ex vivo* model is an attractive proposal for MS study and the assay of potential new therapeutic strategies.

3.1. Novel findings on the role of S100B in the *ex vivo* demyelinated model

Recently, our group showed that S100B is highly released upon LPC-induced demyelination in COSC (Figure I. 4), mostly by astrocytes, in parallel to a massive gliosis (Barateiro et al., 2015).

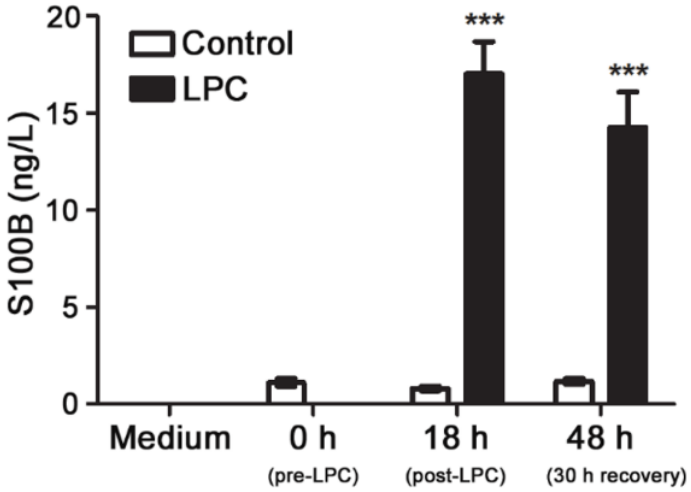

Figure I. 4. S100B is markedly released upon LPC-induced demyelination of cerebellar organotypic slice cultures. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro* during 18 h and allowed to recover for 30 h. Samples for detection of S100B secretion were collected before the incubation (0 h), at 18 h post-incubation with LPC and at 48 h, i.e. after 30 h of recovery. Results are mean ± SEM. ***p<0.001 *vs.* Control. (Adapted from Barateiro et al., 2015).

Curiously, when S100B was therapeutically neutralized using a specific antibody, we could observe a reduced demyelination (Figure I. 5), as well as reduced astrogliosis. Nevertheless, although the density of microglia in the slice showed no apparent differences upon anti-S100B co-treatment, the release of cytokines was markedly reduced suggesting a potential inhibition of microglia pro-inflammatory response (Barateiro et al., 2015).

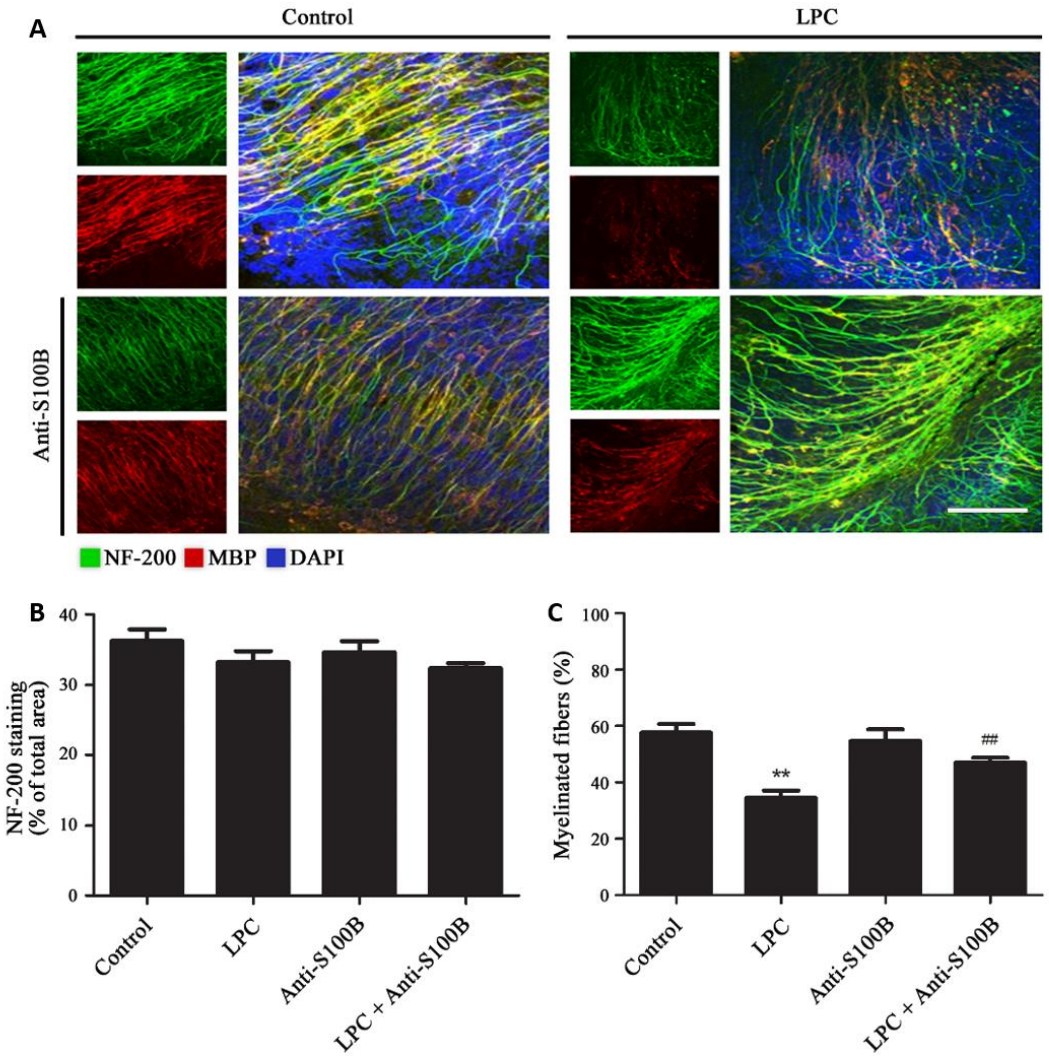

Figure I. 5. Blockade of S100B following demyelination partially prevents loss of myelinated fibers. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro* during 18 h and allowed to recover for 30 h. Slices were double immunostained with neurofilament-200 (NF-200), MBP and DAPI antibodies. **(A)** Confocal images are shown. Scale bar represents 100 µm. **(B)** Quantification of axon integrity was taken by averaging the area occupied by NF-200 staining for each stack. **(C)** The percentage of myelinated fibers was calculated by the ratio between the area of co-localization of NF-200 and MBP and the total area occupied by NF-200. Results are mean ± SEM. **p<0.01 *vs.* Control; ##p<0.01 *vs.* LPC. (Adapted from Barateiro et al., 2015).

So, in preliminary studies we explored the distribution of microglia along the slice. Usually in COSC microglia is distributed along the slice while myelin is found on the top of it. As shown in Figure I. 6, LPC-induced demyelination markedly increase microglia density, namely at the bottom of the slice. However, co-incubation of LPC and anti-S100B, although not preventing the increase of Iba-1 staining, suggest a more evident microglia in the region with highest amount of myelin debris (Figure I. 5). These images suggest that activated microglia moved to myelin surrounds when S100B was blocked, possibly increasing the phagocytosis of myelin debris which is crucial for further remyelination (Afonso, 2014). Since there are recent evidences that microglia switch from a cytotoxic to a protector phenotype during remyelination initiation

(Miron et al., 2013), it would be interest to evaluate if we can accelerate this change of microglia phenotype by neutralizing excessive S100B in the milieu and only preserving it physiological low concentration.

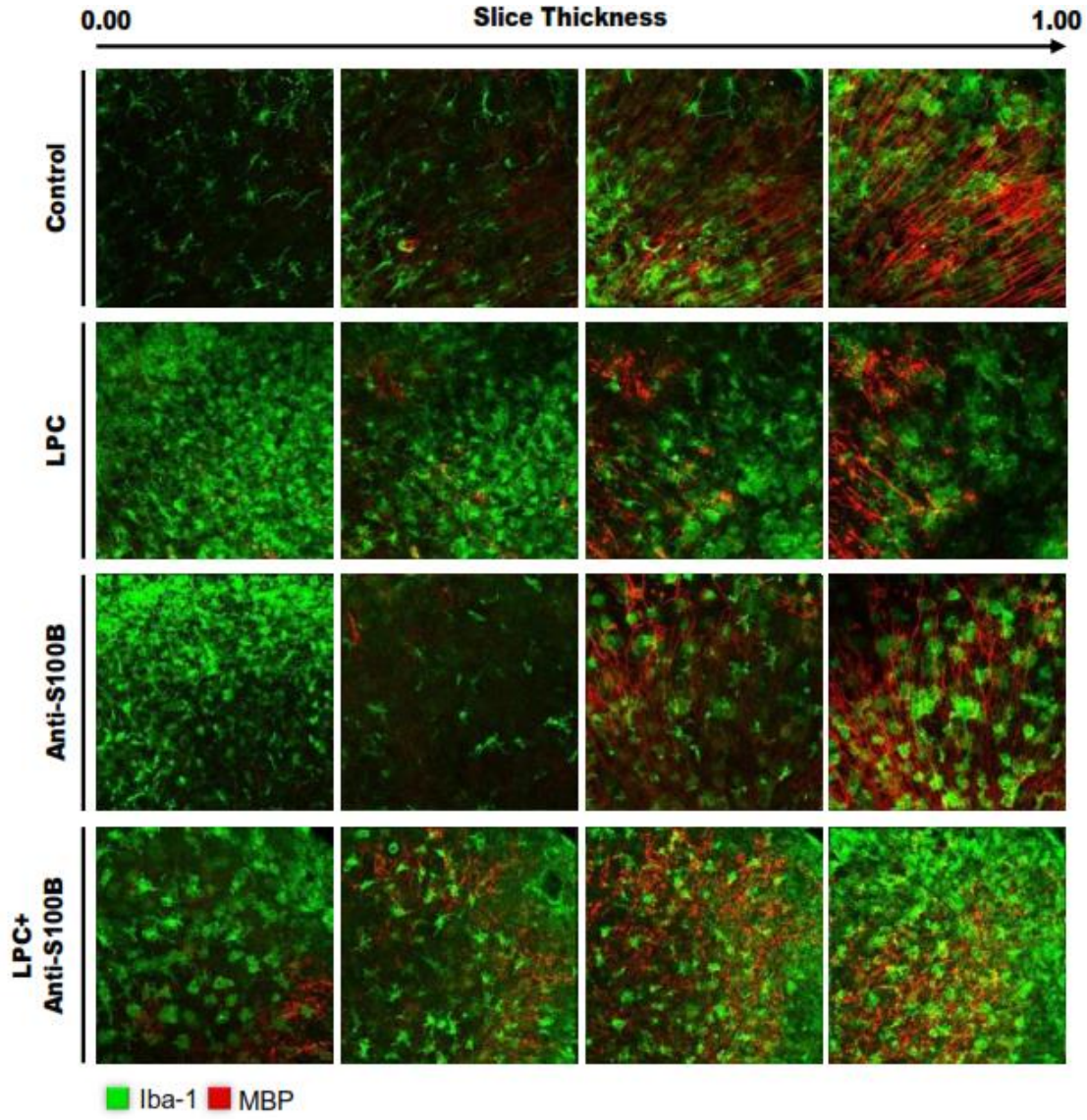

Figure I. 6. Blockade of S100B following demyelination apparently induces microglia migration near to myelin debris. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B antibody, during 18 h and allowed to recover for 30 h. Slices are incubated with Iba-1 and MBP antibodies and analyzed by immunohistochemistry. (Adapted from Afonso V MS thesis).

4. Aims

The principal aim of this work is to evaluate the role of S100B in microglial response during MS. More specifically, we want to understand whether we may promote a more neuroprotective microglia phenotype, during the course of MS, through the modulation of S100B levels.

Therefore, in order to display how S100B modulates microglial reactivity in an *ex vivo* model of demyelination, we will assess (i) the effect of S100B in microglia phenotype-related cell surface receptors; (ii) the role of S100B in microglia phenotype-related markers and (iii) the importance of S100B in microglia phagocytic ability.

II. Material and Methods

1. Animals

CD1 mouse pups were acquired from Instituto Gulbenkian de Ciência (IGC, Lisboa, Portugal). Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in order to use alternatives procedures to *in vivo*.

2. Cerebellar Organotypic Slice Cultures (COSC) and its Treatment

Cerebellum parasagittal slices were obtained from postnatal day 10 (P10) CD1 mouse pups. Briefly, after mice decapitation, the cerebellums were isolated from brains and 400 µm sagittal slices obtained using a Mcllwain tissue chopper. Four slices of different animals were transferred into membrane culture inserts (BD Falcon, #353493, Lincoln Park, NJ, USA) in 6-well cell culture plates that contain 1 mL of medium in per well and kept at 37°C, in 5% CO_2 conditioned atmosphere. Half media was replaced every day and slices were maintained for 7 days *in vitro* (DIV) before treatment, to allow the clearance of debris and full myelination (Birgbauer et al., 2004). The culture medium consisted of 50% minimal essential media (MEM), (Gibco, Life Technologies, Inc., Grand Islands, USA), 25% of both heat-inactivated horse serum (Gibco) and Earle's balanced salt solution (EBSS, Gibco), 6.5 mg/mL glucose, 25mM HEPES (Biochrom AG, Berlin, Germany), 1% of L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 1% of antibiotic-antimycotic (Sigma-Aldrich). After 3 DIV, slice culture media was totally changed by a serum-free media that consists of Neurobasal-A (Gibco) supplemented with 2% B-27 (Gibco), 2 mM L-glutamine, 36 mM glucose, 1% of antibiotic-antimycotic and 25 mM HEPES.

After 7 DIV, culture media was removed and slices were incubated with 0.5 mg/mL LPC in serum-free culture media, during 18h, at 37°C. To evaluate how S100B modulates the microglial reactivity, some slices were also incubated with LPC in the presence of anti-S100B antibody (1:500, AbCam, Cambridge, UK). Additionally, to assess the effect of the presence of an antibody we also incubated the slices with a control IgG (1:500, Santa Cruz Biotechnology, CA, USA). After incubation, the LPC-containing medium was removed and the slices were maintained in fresh medium or in medium supplemented with anti-S100B antibody or IgG during 30h. Following these 30h, slices were: (i) stored in TRIzol® reagent, at -20°C, for RNA extraction; (ii) incubated with ice-cold cell lysis buffer (radio-immunoprecipitation assay buffer – RIPA), for protein extraction; (iii) dissociated and stained with specific antibodies for flow cytometry analysis; (iv) incubated with 1 µm fluorescent latex beads (1:100, Sigma Chemical Co., St. Louis, MO, USA) during 1h, at 37°C, for phagocytic studies; or (v) fixed in 4% paraformaldehyde (PFA) for immunohistochemistry assays. All supernatants were collected both before and after incubation with LPC.

3. Total RNA Extraction, Reverse Transcription, Semi-quantitative RealTime Polymerase Chain Reaction

For the determination of expression levels of diverse genes of interest, total cytoplasmic RNA was isolated from treated slices using the TRIzol® reagent method in accordance with the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA) and RNA concentration was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 500 ng of total RNA were reversely transcribed using the SensiFAST cDNA Synthesis Kit (Bioline), under manufacturer's instructions. Semi quantitative (q)RealTime-PCR was performed on a RealTime PCR detection system (Applied Biosystems 7300 Fast RealTime PCR System, Applied Biosystem, Madrid, Spain) using a SensiFAST SYBR® Hi-Rox Kit (Bioline), under optimized conditions: 50°C for 2 min, 95°C for 2 min followed by 40 cycles at 95°C for 5 s and 62°C for 30 s. In order to verify the specificity of the amplification, a melt-curve analysis was performed immediately after the amplification protocol (95°C for 15 s, followed by 60ºC for 30 s and 95°C for 15 s). The PCR was performed in 96-well plates with each sample performed in duplicate, and a non-template control was included for each gene. The sequences used as primers are listed in the Table II. 1.

Relative mRNA concentrations were calculated using the Pfaffl modification of the $\Delta\Delta C_T$ equation, where C_T is the cycle number at which fluorescence passes the threshold level of

detection, taking into account the efficiencies of individual genes. The results were normalized to the housekeeping gene β-actin in the same sample and the initial amount of the template of each trial was determined as relative expression by the formula $2^{-\Delta\Delta C_T}$. ΔC_T is the value obtained for each sample by performing the difference between the mean C_T value of each gene of interest and the mean C_T value of β-actin. $\Delta\Delta C_T$ of one sample is the difference between its ΔC_T value and the ΔC_T of the sample chosen as reference.

Table II. 1. Primers used for qRealTime PCR

Gene	Forward	Reverse
Arg1	cttggcttgcttcggaactc	ggagaaggcgtttgcttagttc
β-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt
CEBP-α	agcttacaacaggccaggtttc	cggctggcgacatacagtac
CX3CL1	ctcacgaatcccagtggctt	tttctccttcgggtcagcac
CX3CR1	tcgtcttcacgttcggtctg	ctcaaggccaggttcaggag
FIZZ-1	gccaggtcctggaacctttc	ggagcagggagatgcagatgag
HMGB1	ctcagagaggtggaagaccatgt	gggatgtaggttttcatttctctttc
IL-1β	caggctccgagatgaacaac	ggtggagagctttcagctcata
IL-6	ccggagaggagacttcacag	ggaaattggggtaggaagga
IL-18	tggttccatgctttctggactcct	ttcctgggccaagaggaagtg
iNOS	acccacatctggcagaatgag	agccatgacctttcgcattag
MBP	ccatccaagaagaccccaca	cccctgtcaccgctaaagaa
MHC-II	tgggcaccatcttcatcattc	ggtcacccagcacaccactt
NLRP3	tgctcttcactgctatcaagccct	acaagcctttgctccagaccctat
SOCS-1	caccttcttggtgcgcg	aagccatcttcacgctgagc
TGF-β	cagagctgcgcttgcagag	gtcagcagccggttaccaag
TLR2	tgctttcctgctgaagattt	tgtaccgcaacagcttcagg
TLR4	acctggctggtttacacgtc	gtgccagagacattgcagaa
TNF-α	tactgaacttcggggtgattggtcc	cagccttgtcccttgaagagaacc

4. Protein Extraction and Western Blot

For the determination of cellular protein content, proteins were obtained by lysing cells in ice-cold cell lysis RIPA buffer, followed by sonication and centrifugation at 12,000 g for 10 min. Total protein concentrations were always measured using the Bradford method with Bio-Rad's Protein Assay Reagent (BioRad Laboratories, Hercules, CA, USA).

Protein samples were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were incubated in blocking buffer [Tween 20 (0.1%)-Tris buffered saline, T-TBS, plus 5% (w/v) non-fat dried milk] at room temperature during 1 hour and, after blocking, they were incubated overnight at 4°C, with primary antibodies (Table II. 2), diluted in T-TBS with 5% BSA. After three washes with T-TBS, the membranes were incubated at room temperature during 1 hour with respective secondary antibodies, specified in Table II. 3, diluted in blocking solution. After washing membranes with T-TBS, chemiluminescent detection was performed using LumiGLO® reagent (Cell Signalling, Beverly, MA, USA), bands were visualized in Chemidoc equipment and relative intensities of protein bands were analyzed using the Image Lab analysis software, both from Bio-Rad Laboratories (Hercules, CA, USA). The results were normalized to the housekeeping protein β-actin.

Table II. 2. Primary antibodies used for immunoblot assays

Antibody	Host	Brand	Reference Number	Dilution
β-actin	Mouse	Sigma-Aldrich	A5441	1:5000
HMGB1	Mouse	BioLegend	651402	1:100
TLR4	Rabbit	Santa Cruz Biotechnology	sc-10741	1:100

Table II. 3. Secondary antibodies used for immunoblot assays

Antibody	Brand	Reference Number	Dilution
Horseradish-peroxidase-conjugated anti-mouse	Santa Cruz Biotechnology	sc-2032	1:5000
Horseradish-peroxidase-conjugated anti-rabbit	Santa Cruz Biotechnology	sc-2004	1:5000

5. Flow Cytometry – Fluorescence-activated cell sorting

Slices were collected and incubated with 100 mg/mL of collagenase (1:100) during 10 min, at 37°C, with agitation. Then, slices were dissociated and cell suspension made in flow buffer (PBS plus 2% FBS and 0.02% sodium azide). To prevent non-specific binding, cells were incubated for 20 min with CD16/CD32 (1:100) to block Fc receptors, at 4°C. After, cell suspension was incubated with the fluorescent labeled antibodies CD11b PerCp-Cy5, F4/80 FITC, CD45 PE and CD86 Bio-SAV PE for 30 min, at 4°C (1:100). Following the incubation, cells were washed with flow buffer, incubated with streptavidin (1:100) for the CD86 Bio-SAV PE antibody during

30 min, and then resuspended in flow buffer. Expression of surface antigens was measured using the BD FACSCalibur flow cytometer and data analyzed using the FlowJo software.

6. Immunostaining procedure for phagocytic analysis

For immunostaining procedure, insert membranes, which contain the fixed slices, were cut, placed into a cover glass and blocked with 1nM HEPES, 2% heat-inactivated horse serum, 10% heat-inactivated goat serum (Biochrom), 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.25% Triton X-100 (Roche Diagnostics, Indianapolis, USA) in Hank's balanced salt solution (HBSS, Gibco) for three hours, at room temperature. After blocking, slices were incubated with primary antibody (Iba-1, 1:250, Wako) diluted in the blocking solution, for 24h, at 4°C. After incubation, slices were washed three times for 15 min each with 0.01% Triton X-100 in PBS (T-PBS) and incubated with secondary antibody (Alexa 594 anti-rabbit, 1:1000 Invitrogen) in blocking solution, for 24 h, at 4°C. Slices were then washed three times for 15 min each with T-PBS, incubated with DAPI (1:1000, 3 min), washed three times for 15 min each with T-PBS and mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) for confocal microscopy. Fluorescent images were acquired using a Leica DM6000 inverted microscope and analyzed with ImageJ software. For each image were determined the total number of phagocyting cells and the total number of internalized beads.

7. Statistical analysis

Results are presented as mean ± SEM. Differences between groups were determined by one-way ANOVA using GraphPad PRISM 5.0 (GraphPad Software, San Diego, CA, USA), as appropriate. The *p*-values lower than 0.05 were considered as being statistically significant.

III. Results

1. Myelin-related protein expression is recovered after S100B blockage

Knowing that myelin-related MBP is an important constituent of myelin sheaths and to show that our *ex vivo* model is a good model to study demyelinated lesions, we decided to evaluate the gene expression of this protein extracted from COSC at 48 h post-incubation with LPC, by qRealTime PCR, using specific primers.

As illustrated in Figure III. 1, LPC insult promoted a significant decrease in MBP and PLP expression (0.36-fold, p<0.001). Interestingly, co-incubation with anti-S100B antibody markedly prevented this MBP reduction (~80%, p<0.001), while the co-treatment with IgG did not significantly changed MBP mRNA expression.

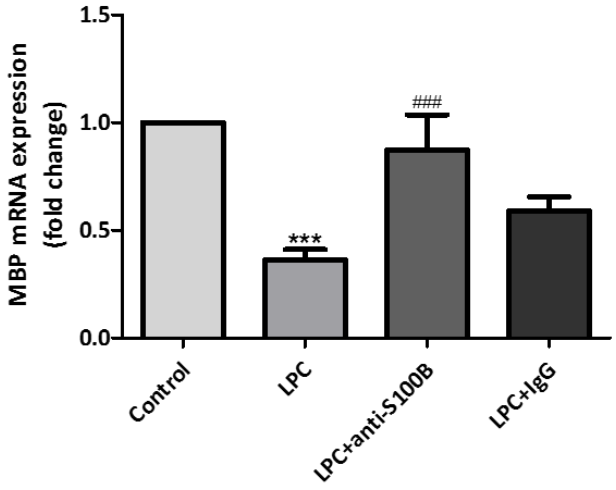

Figure III. 1. S100B neutralization attenuates demyelination induced by LPC. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Gene expression of MBP was evaluated at 48 h by qRealTime PCR. Results are mean ± SEM. ***p<0.001 *vs.* Control; ###p<0.001 *vs.* LPC

2. Neutralization of S100B prevents a microglia pro-inflammatory phenotype

Along with demyelination, MS lesions are also characterized by microgliosis (Dyer et al., 2005). Knowing that the inhibition of S100B does not prevent the increase of microglia density although it prevents the release of cytokines on our *ex vivo* demyelinating model (Barateiro et al., 2015), we decided to characterize the populations of reactive microglia in our COSC at 48 h post-LPC, by flow cytometry. Generally it is accepted that pro-inflammatory activated microglia is characterized as CD11b^{high}, CD86^{high}, CD45$^{dim\ hi}$ and F4/80^{high} staining (Devarajan et al., 2014).

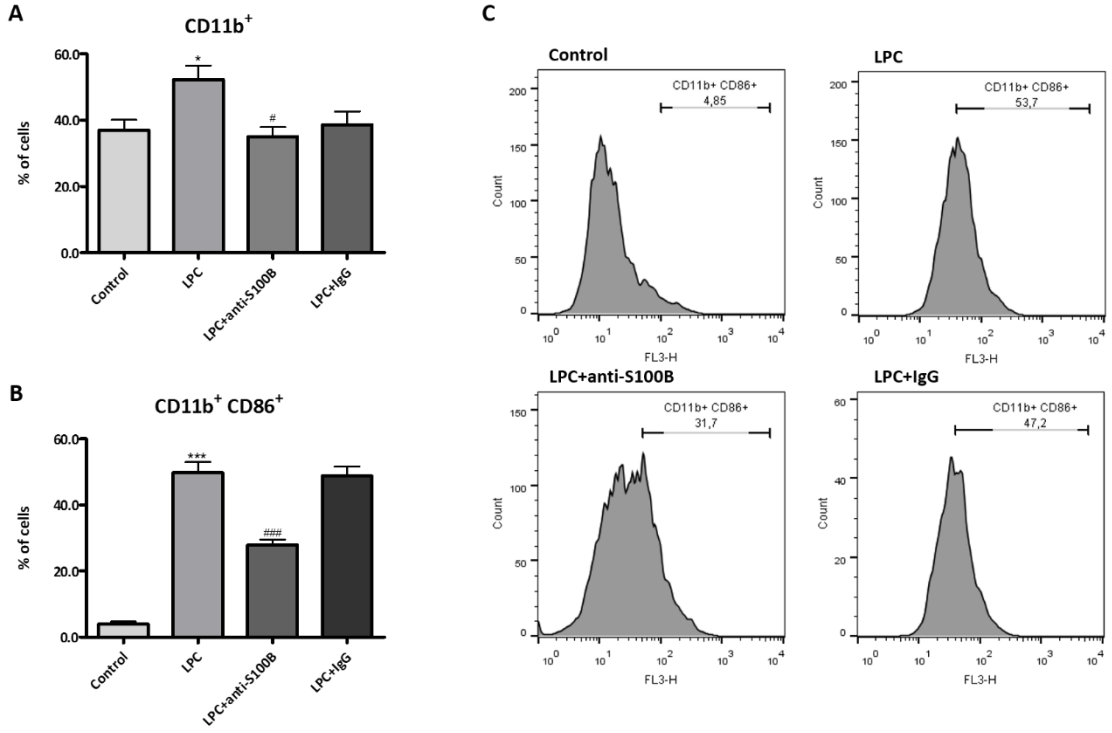

Figure III. 2. S100B abrogation significantly decreases CD11b$^+$/CD86$^+$ microglia population induced by LPC-demyelination. Cerebellar organotypic slice cultures were treated with LPC, at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Dissociated slices were stained with CD11b- and CD86-specific monoclonal antibodies and analyzed by flow cytometry. **(A)** Data are expressed as mean of percentage of CD11b$^+$ and **(B)** CD11b$^+$/CD86$^+$ cells ± SEM. ***p<0.001 and *p<0.05 *vs.* Control; $^{###}$p<0.001 and $^{#}$p<0.05 *vs.* LPC. **(C)** Representative images of cytograms are showed.

As shown in Figure III. 2 A, treatment with LPC induced an increase of the population of more reactive microglia expressing CD11b (from 36.99% to 52.26%, p<0.05), which is prevented by anti-S100B neutralization. Next, we analyzed which CD11b$^+$ population also expressed the pro-inflammatory cell surface receptors CD86, CD45 and F4/80. Figure III. 2 B clearly shows that LPC treatment increases the number of CD11b$^+$/CD86$^+$ microglia (from 3.88% to 49.75%, p<0.001), which is partially prevented by S100B neutralization by ~50% (p<0.001), while IgG had

no effect. These results show that, blockade of S100B may prevent a pro-inflammatory phenotype, as demonstrated by a significant reduction on $CD11b^+/CD86^+$ microglia population. On the other hand, we saw no differences between control and LPC, or even anti-S100B treatments in the other markers (Table III. 1), suggesting that demyelination did not influence their expression.

Table III. 1. Demyelination or antibodies treatment on cerebellar organotypic slice cultures do not change the percentage of CD11b-positive cells that express F4/80 and CD45 markers.

	$CD11b^+$ $F4/80^+$	**$CD11b^+$ $CD45^{2+}$**
Control	63.06 % ± 8.04 %	16.03 % ± 7.01 %
LPC	65.56 % ± 7.24 %	20.58 % ± 7.25 %
LPC + anti-S100B	60.08 % ± 7.07 %	24.78 % ± 5.97 %
LPC + IgG	61.86 % ± 6.94 %	22.25 % ± 7.05 %

Cerebellar organotypic slice cultures were treated with LPC, at 7 days in vitro, in presence or absence of S100B or IgG antibodies, during 18 h and allowed to recover for 30 h. Dissociated slices were stained with CD11b-, F4/80- and CD45-specific monoclonal antibodies and analyzed by flow cytometry. Data are expressed as mean of percentage of positive cells ± SEM.

3. Abrogation of S100B shifts microglia from a pro-inflammatory phenotype to a more neuroprotective one

As mentioned above, MS lesions are characterized by microglia activation that lead to the secretion of pro-inflammatory cytokines and inflammasome-related proteins production that enhance the inflammatory response, contributing to disease progression (Benarroch, 2013; Vainchtein et al., 2014). Moreover, NLRP3 inflammasome, a complex involved in the maturation and secretion of pro-inflammatory molecules, have recently been associated with MS pathology. Once we have shown that S100B inhibition seems to prevent a pro-inflammatory response from microglia, we decided to explore the expression of these several inflammatory mediators upon demyelination and in the presence of anti-S100B antibody.

As shown in Figure III. 3, LPC-induced demyelination increased the expression of cytokines TNF-α and IL-1β but decreased the IL-6 expression (1.46-, 6.24- and 0.35-fold, $p<0.01$ and $p<0.001$, respectively). Anti-S100B treatment partially prevented LPC-induced alterations in cytokine expression (~56.52%, 63.17% and 76.92%, $p<0.01$, respectively), while no significant changes was observed upon IgG treatment. These results confirm the stimulation of an

inflammatory response upon demyelination and suggesting the involvement of S100B in cytokine release by microglial cells.

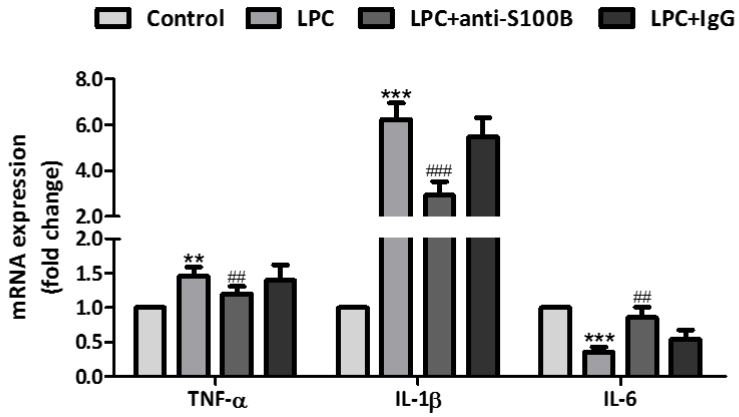

Figure III. 3. S100B neutralization prevents the increase of TNF-α and IL-1β expression and the inhibition of IL-6 expression induced by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Gene expression of TNF-α, IL-1β and IL-6 were evaluated at 48 h by qRealTime PCR. Results are mean ± SEM. ***p<0.001 and **p<0.01 *vs.* Control; ###p<0.001 and ##p<0.01 *vs.* LPC.

Inflammasomes are cytosolic sensors that detect alterations and are involved in maturation and release of pro-inflammatory cytokines, such as HMGB1, IL-1β and IL-18 (Inoue and Shinohara, 2013; Lu et al., 2013). The NLRP3 inflammasome consists of three proteins that has already been associated with MS development (Inoue and Shinohara, 2013). As we have previously observed, a marked increase of IL-1β expression upon demyelination and its protection after S100B inhibition, we next evaluated the gene expression of the other inflammasome-associated molecules.

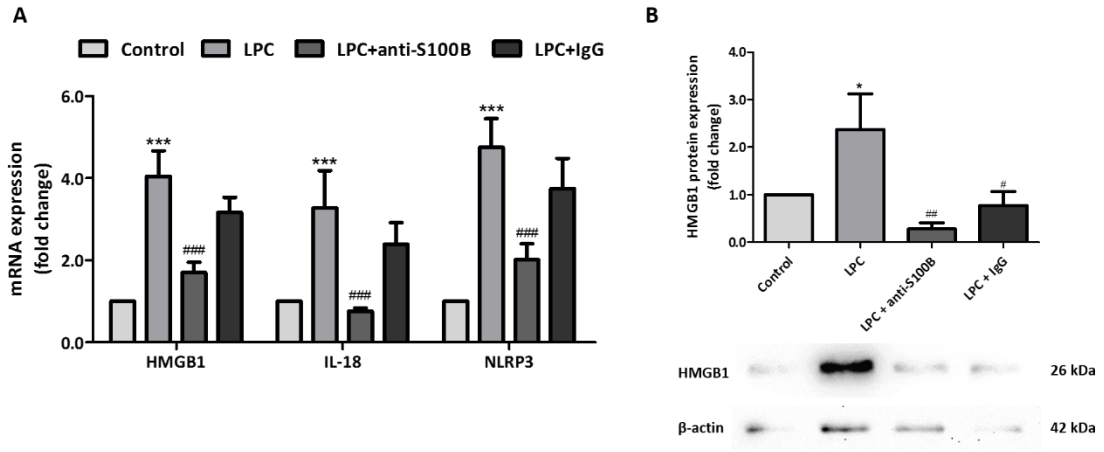

Figure III. 4. S100B neutralization prevents NLRP3 activation and inflammasome-related molecules expression induced by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. **(A)** Gene expression of HMGB1, IL-18 and NLRP3 were evaluated at 48 h by qRealTime PCR. **(B)** Protein expression of HMGB1 was evaluated at 48 h by Western Blot. Results are mean ± SEM. ***p<0.001 and **p<0.01 *vs.* Control and ###p<0.001, ##p<0.01 and #p<0.05 *vs.* LPC.

LPC markedly increased the expression of NLRP3 (4.75-fold, p<0.001), as well as that of cytokines that are processed by the inflammasome, HMGB1 and IL-18 (4.05- and 3.28-fold, p<0.001, respectively), as depicted in Figure III. 4 A. Neutralization of S100B prevented the expression of all inflammasome-related proteins (77.05%, 110.53% and 73.07%, p<0.001, for HMGB1, IL-18 and NLRP3, respectively). We next confirm whether protein expression was also altered in the same way for HMGB1. As demonstrated in Figure III. 4 B, HMGB1 was increased upon demyelination (2.37-fold, p<0.05) and, co-incubation with anti-S100B antibody reduced HMGB1 protein expression by 152.55%, p<0.01, although IgG incubation also seemed to have partial effect in these protein levels. All these results corroborate that we may decrease the inflammatory environment related to microglia activation with S100B neutralization.

Since we observed a decrease in the release of pro-inflammatory molecules in the presence of S100B at physiological levels, we then looked at markers usually identified in M1 pro-inflammatory microglia phenotype.

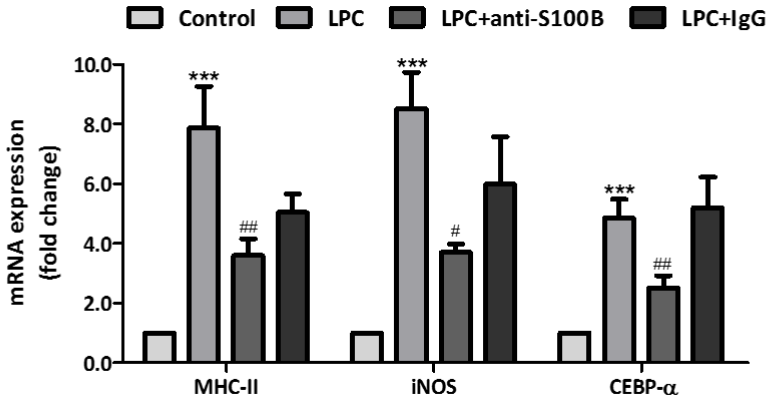

Figure III. 5. S100B neutralization prevents the increase of MHC-II, iNOS and CEBP-α expression induced by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Gene expression of MHC-II, iNOS and CEBP-α were evaluated at 48 h by qRealTime PCR. Results are mean ± SEM. ***p<0.001 *vs.* Control; ##p<0.01 and #p<0.05 *vs.* LPC.

LPC-induced demyelination promoted a marked increase of gene expression of M1 cell surface markers MHC class II, iNOS and CEBP-α (7.87-, 8.51- and 4.85-fold, p<0.01, respectively), as presented in Figure III. 5. Interestingly, S100B blockade was able to prevent these expression for more than 60% (p<0.05), indicating a reduced M1 microglia polarization when elevated levels of S100B are neutralized in the culture medium. Antiserum treatment partially reduced MHC-II and iNOS expression suggesting a possible action at this markers.

Microglia are rapidly activated in the CNS in response to several injuries, including inflammation. TLRs, an important member of pattern recognition receptors family, are

considered to be involved in MS pathology. Since there are studies showing the elevated expression of both TLR2 and TLR4 in MS patients (Miranda-Hernandez and Baxter, 2013), we decided to analyze the gene expression of these receptors.

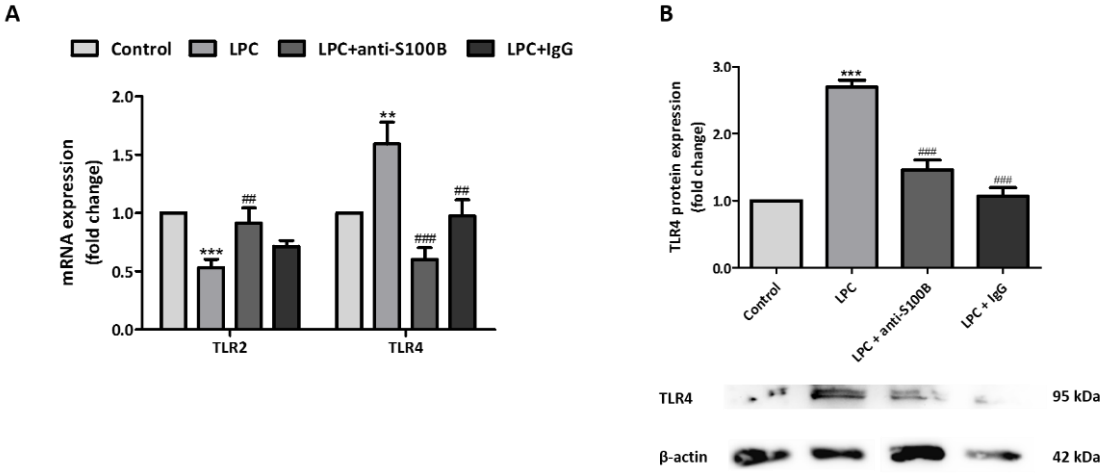

Figure III. 6. S100B inhibition prevents the alterations of TLR2/4 expression induced by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. **(A)** Gene expression of TLR2 and TLR4 were evaluated at 48 h by qRealTime PCR. **(B)** Protein expression of TLR4 was evaluated at 48 h by Western Blot. Results are mean ± SEM. ***p<0.001 and **p<0.01 *vs.* Control; ###p<0.001 and ##p<0.01 *vs.* LPC.

Accordingly with previous studies, both mRNA and protein TLR4 levels were increased upon demyelination (1.59- and 2.69-fold, p<0.01, respectively) possibly indicating an attempt of microglia to respond to an inflammatory stimulus (Figure III. 6 A, B). S100B inhibition prevented both LPC-induced TLR4 gene and protein expression (179.66% and 72.78%, p<0.001, respectively). However, also IgG co-incubation decreased TLR4 expression suggesting a potential effect of the antiserum. Concerning TLR2, its gene expression was decreased by LPC-induced demyelination (0.53-fold, p<0.001), what was abrogated by anti-S100B co-incubation to control levels (p<0.01). These results indicating that the inflammatory response may not occur through TLR2 pathway (Figure III. 6 A).

Having confirmed the reduction of a pro-inflammatory phenotype with S100B inhibition, and knowing that microglia are the most responsible for inflammatory cytokine production, we evaluated whether microglia could be shifting from a M1 to a M2 phenotype with S100B blockade. In this regard, we analyzed COSC lysates 48 h after LPC treatment for different M2-like gene expression.

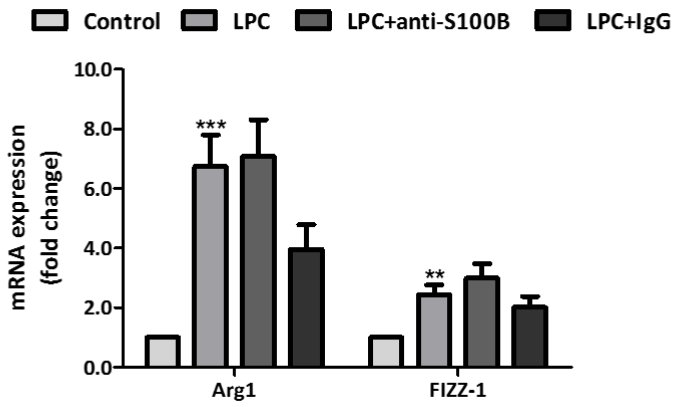

Figure III. 7. S100B neutralization slightly intensifies the increase of Arg1 and FIZZ-1 expression induced by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Gene expression of Arg1 and FIZZ-1 were evaluated at 48 h by qRealTime PCR. Results are mean ± SEM. ***p<0.001 and **p<0.01 *vs.* Control.

So first we looked at Arg1 and FIZZ-1 expression (Figure III. 7), LPC-induced demyelination markedly increased these markers (6-75- and 2.45-fold for Arg1 and FIZZ-1, $p<0.01$, respectively), corroborating previous studies that show an increased M2 phenotype when remyelination is beginning (Miron et al., 2013).

Neutralization of S100B did not significantly change the expression of both Arg1 and FIZZ-1 compared to LPC treated COSC (7.08- and 2.99-fold for Arg1 and FIZZ-1, respectively), suggesting that blockade of excessive amounts of S100B did not increase a microglia shift to a M2 phenotype increase. On the other hand, when other M2 microglial markers were analyzed, such as TGF-β and SOCS-1 (Figure III. 8), LPC-induced demyelination still markedly increased these markers (2.40- and 15.13-fold for TGF-β and SOCS-1, $p<0.01$, respectively), while S100B neutralization clearly decreased their expression to near control levels (0.86- and 1.83-fold for TGF-β and SOCS-1, $p<0.01$, respectively). Collectively, these results suggest that neutralization of S100B although preventing an exacerbated M1 phenotype microglia does not promote the shift of that microglia to a M2 phenotype. Curiously, also in here IgG co-incubation had an effect, reducing LPC-induced M2-markers expression but with a different trend than anti-S100B, indicating that although the antiserum may have an effect it does not completely mask the real anti-S100B action.

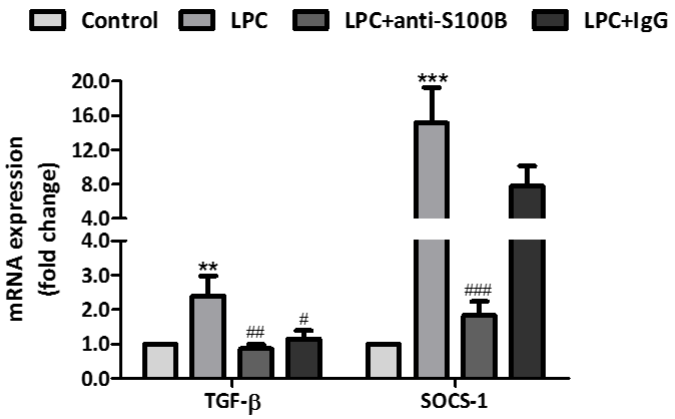

Figure III. 8. Inhibition of S100B neutralization diminished TGF-β and SOCS-1 expression induced by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Gene expression of TGF-β and SOCS3 were evaluated at 48 h by qRealTime PCR. Results are mean ± SEM. ***p<0.001 and **p<0.01 *vs.* Control; ###p<0.001, ##p<0.01 and #p<0.05 *vs.* LPC.

Collectively, these results suggest that neutralization of S100B although preventing an exacerbated M1 phenotype microglia does not promote the shift of that microglia to a typical M2 phenotype, but may reduce their activation state due to a less demyelinated and inflammatory milieu.

4. Neutralization of S100B change microglia phagocytic ability

An important neuroprotective role of microglial cells is their ability to phagocyte, playing a crucial role in CNS both in pathology and tissue regeneration (Goldmann and Prinz, 2013; Kettenmann et al., 2011). Therefore, we explored the effect of S100B in microglia phagocytic properties by microglia immunohistochemistry with Iba-1 antibody following exposure to fluorescent zymosan-coated beads.

As illustrated in Figure III. 9, treatment with LPC decreased both the number of phagocytosed beads per cell (0.49-fold, p<0.001) and the number of phagocyting microglia (0.41-fold, p<0.01). Interestingly, results show that there are an increase in the number of microglial cells that phagocyte after co-incubation of LPC with anti-S100B (1.00-fold, p<0.01), while the number of cells phagocyting a higher number of beads is also increased by S100B neutralization when different cutoffs of beads are analyzed (Figure III. 9 B), namely for less than 20 beads. However, once again we see a partial effect also for IgG co-incubation.

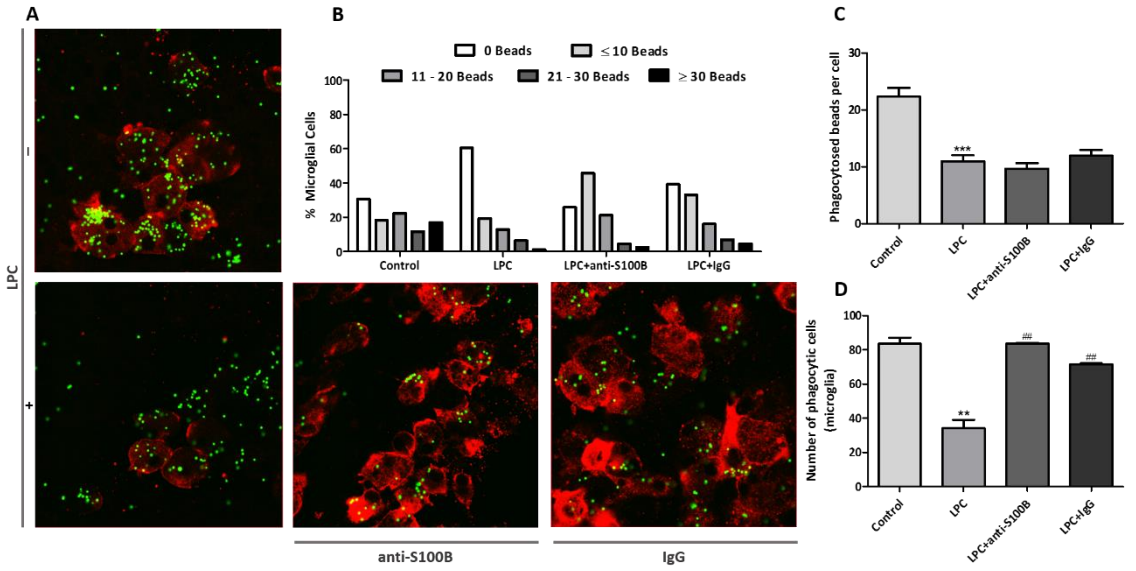

Figure III. 9. Blocking S100B increases the number of phagocytic cells following demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Slices are incubated with fluorescent latex beads to measure the phagocytic ability of microglial cells, by immunohistochemistry. **(A)** Confocal images showing the ingestion of the fluorescent beads (green) by Iba-1 (red) positive microglia in COSC. **(B)** Number of microglia phagocytosing 0, ≤10, 11-20, 21-30, and >30 beads, **(C)** number of phagocytosed beads per cell and the **(D)** number of microglial cells that phagocyte were counted. Results are mean ± SEM. ***p<0.001 and **p<0.01 *vs.* Control; ##p<0.01 *vs.* LPC.

5. S100B inhibition tries to recover the neuron-microglia communication

Fractalkine (CX3CL1) has a role in specific communication with microglia, the only cells in the CNS that express the correspondent receptor, CX3CR1 (Harrison et al., 1998). Fractalkine is highly expressed in neurons, in the normal brain, allowing the hyporesponsiveness of microglia due to its ability to inhibit microglial activation. Loss of fractalkine or its receptor expression could impair this neuron-microglia dialogue leading to microglial activation (Zujovic et al., 2000). Thus, and considering that the fractalkine/CX3CR1 signaling plays a role in neuroinflammatory and autoimmune diseases of the CNS, we next explored the expression of fractalkine and CX3CR1 upon demyelination and in the presence of anti-S100B antibody to understand the role of S100B in this dialogue.

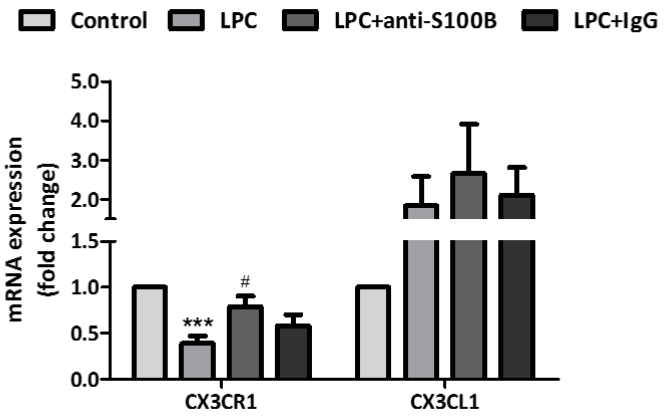

Figure III. 10. S100B neutralization tries to recover the neuron-microglia communication lost by LPC-demyelination. Cerebellar organotypic slice cultures were exposed to LPC at 7 days *in vitro*, in presence or absence of S100B and IgG antibodies, during 18 h and allowed to recover for 30 h. Gene expression of CX3CR1 and CX3CL1 were evaluated at 48 h by qRealTime PCR. Results are mean ± SEM. ***p<0.001 *vs.* Control. #p<0.05 *vs.* LPC.

As depicted in Figure III. 10, treatment with LPC decreased the expression of fractalkine receptor CX3CR1 when compared with control (0.39-fold, $p<0.001$) rendering cells less responsive to CX3CL1, while increased the expression of the chemokine itself. Interestingly, CX3CR1 reduction is attenuated after S100B abrogation (~66%, $p<0.05$), whereas the expression of CX3CL1 is slightly increased after anti-S100B antibody co-incubation, suggesting an attempt to restore this mechanism of microglia deactivation.

IV. Discussion

S100B is associated with different neurodegenerative pathologies, including MS. Indeed, increased levels of S100B have been detected in both CSF and *post-mortem* demyelinated plaques of MS patients. The development of these demyelinated lesions, one hallmark of MS, are a result of several mechanisms including gliosis, inflammation, demyelination, axonal loss and neuronal degeneration (Milo and Miller, 2014). Thus, with this thesis we proposed to study the role of S100B in MS associated microglia reactivity, using an *ex vivo* model of demyelination (Birgbauer et al., 2004). Firstly, we evaluated whether the expression of a protein constituent of myelin sheaths could be recovered, in the course of the demyelinating insult with LPC by neutralization of excessive S100B. Our results revealed that the expression of MBP mRNA was decreased after demyelinating insult, according with Birgbauer and collaborators, which reported a significant decrease in myelin marker expression (Birgbauer et al., 2004). Interestingly, treatment with anti-S100B antibody prevented this demyelination, as previously reported by us (Barateiro et al., 2015), increasing the MBP mRNA expression. These data suggest that the neutralization of S100B prevented an exacerbated demyelination by keeping S100B concentration close to physiological levels and, therefore, having a protective role on demyelinating event.

In healthy CNS, microglial cells, which are constantly patrolling the parenchyma, are characterized by a ramified morphology and low levels of CD11b and F4/80, macrophage/microglia activation marker, and CD45 and CD86, M1 markers (Devarajan et al., 2014; Ponomarev et al., 2013). As we known, microglia become active during CNS inflammation, acquiring phenotypic and morphologic changes in response to inflammation. These activated microglia are generally characterized as $CD11b^{high}/CD86^{high}/F4/80^{high}$ and $CD45^{dim\ high}$ (Devarajan et al., 2014; Ponomarev et al., 2005, 2013). It is difficult to differentiate CNS resident microglia from peripheral macrophages because they present a similar pattern, however, our demyelinating *ex vivo* model does not involve these peripheral type of cells. Accordingly with

previous studies, we observed an increase of activated microglia population upon demyelination, observed through CD11b augment, which suggests their increased ability to respond to an injury. Once having confirmed the activation of microglia after a demyelinated insult, we evaluated their phenotype analyzing the expression of M1 markers and we only observed an upregulation of CD86 in response to LPC stimulation. Notably, the effect of S100B neutralization diminished CD86 cell surface expression suggesting a reduction of this pro-inflammatory phenotype. Ponomarev and collaborators, reported a decrease in the expression of reactive marker CD11b when microglia acquire the M2 phenotype (Ponomarev et al., 2013), our results showed that neutralization of S100B promoted a reduction in CD11b levels expression suggesting a shift of microglia phenotype into a more anti-inflammatory one. In turn, we also analyzed other M1 markers, such as F4/80 and CD45, which did not statistically differ between conditions, indicating that in our demyelinating *ex vivo* model those are not the best markers to detect changes in microglia phenotype. Moreover, the population of microglia expressing CD45 was very low, corroborating the concept that the expression of this marker may distinguish between $CD45^{dim\ hi}$ microglia from $CD45^{high}$ macrophages.

Although they are often referred to have a "resting" phenotype under healthy conditions, microglia cells are constantly patrolling their milieu and, therefore, their activation due to any damage, may contribute to an inflammatory environment by the secretion of inflammatory factors. According to Donato and collaborators which reported an increase of pro-inflammatory cytokines release with high S100B levels (Donato et al., 2013), our results showed an increase in the expression of first-line cytokines TNF-α and IL-1β upon a demyelinated insult, in parallel to an increased S100B expression as previously reported by us (Barateiro et al., 2015). Moreover, our data also showed that S100B blockade prevents this cytokine increase. Once these cytokines are mainly released by activated microglia, these results suggest a microglial shift from a pro-inflammatory phenotype to less inflammatory one. In addition, our results showed a demyelination-induced decrease in pro-inflammatory IL-6 mRNA expression that returned to control levels following S100B neutralization. In spite of the large number of studies that reported the role of IL-6 in MS pathology, there is still a great controversy. As already mentioned, several studies reported an increase in IL-6 mRNA expression in chronic lesions (Maimone et al., 1997; Woodroofe and Cuzner, 1993), CSF (Navikas et al., 1996) and in peripheral blood monocytes (Frei et al., 1991) from MS patients, showing that this cytokine is involved in MS progression. However, there are studies that particularly shown low IL-6 levels in chronic inactive lesions and in plaques with oligodendrocyte loss (Schönrock et al., 2000),

suggesting that the reduced IL-6 expression upon demyelination, observed in our results, may be associated with oligodendrocyte damage/death (Bø et al., 2013).

During MS pathophysiology microglia are activated in response to demyelination and release different inflammatory cytokines. Recently, some studies suggest the involvement of NLRP3 inflammasome on MS and EAE progression (Inoue and Shinohara, 2013). As mentioned above, inflammasomes are involved in maturation and release of cytokines. Indeed, IL-1β, HMGB1 and IL-18 are cytokines matured by NLRP3 inflammasome. HMGB1, a cytokine involved in the start of CNS inflammatory response, is increased in MS and EAE lesions (Andersson et al., 2008). IL-18, a pro-inflammatory cytokine has also been revealed to be increased in MS patients (Sedimbi et al., 2013). Corroborating these studies, our results showed an increased expression of NLRP3, HMGB1 and IL-18 upon LPC-induced demyelination. Interestingly, there are a manifest reduction of inflammasome-related molecules by neutralization of S100B, indicating that excessive S100B expression following demyelination is not only exacerbating cytokine expression but also their maturation into active forms.

In the activated state, microglia exhibit an upregulation of several markers according to the acquired phenotype. Particularly, in response to demyelination, pro-inflammatory microglia may increase the expression of several inflammatory markers, including MHC-II, iNOS and CEBP-α. It has been demonstrated that classically activated microglia increased the expression of CEBP-α after brain injury, indicating that this transcription factor may regulate microglial function during brain damage (Walton et al., 1998). Also, Ponomarev and collaborators showed, in EAE model, an augmented expression of CEBP-α associated with the upregulation of other M1 markers, such as MHC-II and iNOS (Ponomarev et al., 2011). Our *ex vivo* model also showed an increase of these pro-inflammatory markers in response to LPC thus corroborating these findings. Interestingly, the inhibition of excessive S100B in the milieu appears to be effective in the prevention of the expression of these cytokines, corroborating once again that S100B blockade reduces M1 pro-inflammatory microglia phenotype.

MS is a chronic disorder where activated microglia play an important immune response. Microglia sensor danger/stranger signals activating the TLRs (Glass et al., 2010). Once TLRs are engaged, it initiates a cascade of events culminating into the activation of the transcription factor NF-κB and contribute to inflammation (Kettenmann et al., 2011). In this context, TLRs have been shown to be implicated in several CNS diseases including MS (Miranda-Hernandez and Baxter, 2013). Our results showed an augment of TLR4 but not of TLR2 gene expression upon demyelinated conditions, indicating that in our model TLR4 is possibly more responsible for microglia activation than TLR2. Moreover, the treatment with anti-S100B prevented the

increase of TLR4 and maintained the TLR2 expression at control levels, indicating that excessive S100B may also be exacerbating inflammation through TLR4.

In turn, anti-inflammatory microglia, usually found in initial remyelination, are characterized by upregulated Arg1, FIZZ-1, TGF-β and SOCS-1, amongst many other markers (Chhor et al., 2013; Correale, 2014; David and Kroner, 2011; Hu et al., 2014; Ponomarev et al., 2013; Wilson, 2014). In accordance with these previous studies, 48 h after a demyelinating insult there are a marked upregulation of Arg1 and FIZZ-1 gene expression. Curiously, S100B neutralization did not significantly changes these levels suggesting a maintenance of M2 phenotype in the presence of low levels of S100B protein. However, TGF-β and SOCS-1 expression was almost completed abrogated by the inhibition of S100B. Besides the important immunosuppressive role of TGF-β described in relapsing EAE and MS, where it prevents T cells from entering into CNS, there are evidences that this cytokine can increase Th17 production in inflammatory tissues further promoting pro-inflammatory gene expression in CNS tissue (Mirshafiey and Mohsenzadegan, 2009). So, the reduction of TGF-β levels observed by S100B neutralization may suggest an important involvement of S100B in Th17 response and recruitment to the CNS, which should be further elucidated in *in vivo* systems. In addition, despite of microglia phenotypes have been considered separable stages, different populations with distinct phenotypes are simultaneously present upon inflammatory conditions. As already mentioned, SOCS-1 inhibits the expression of pro-inflammatory markers at high levels (Davey et al., 2006). In addition, as we already demonstrated, there is an increase of several pro-inflammatory markers expression upon demyelination. However, in accordance with Kakhki and collaborators that showed an increase of SOCS-1 expression in MS patients, our results revealed that SOCS-1 mRNA was overexpressed in a demyelinated condition (Kakhki et al., 2015). Thus, the fact that there is an increased expression of SOCS-1 after the incubation with LPC may indicate a response from microglia to fight the high inflammatory burden.

Phagocytosis is a crucial mechanism for clearance of pathogens, dying cells and debris in order to maintain tissue health. Microglia are able to phagocyte either debris, including myelin debris, and apoptotic cells that are internalized through phosphatidylserine receptors, stimulating an anti-inflammatory response; or infectious pathogens through TLRs, inducing the release of pro-inflammatory cytokines (Napoli and Neumann, 2010). While M1 microglia exert cytotoxic effects on neurons, M2 microglia exhibit phagocytic capacity promoting neurite outgrowth (Prinz and Priller, 2014). Here, we investigated the ability of microglia to phagocyte zymosan-coated latex beads, which would mimic the phagocytosis of a pathogen. LPC treatment reduced both the number of phagocytosed beads per cell as well as the number of phagocyting

microglia. These results may suggest that either microglia ability to phagocyte is reduced by LPC-induced demyelination or that in the presence of demyelination, microglia has to phagocyte myelin debris and are no longer able to further phagocyte the beads to which they are exposed at the end of the 48 h post-LPC. Curiously, although S100B inhibition did not change the number of beads that microglia can phagocyte, we verified an increase of microglial cells that phagocytosed less beads. This data may suggest that microglia may show an enhanced phagocytic ability upon neutralization of excessive S100B or that there is less myelin debris to phagocyte and therefore microglia are more prone to zymosan-coated beads. To that, it would be interesting to investigate the co-localization of myelin and microglia in our model of demyelination to analyze the amount of myelin debris that have been already phagocytosed by microglia during culture period to better clarify this issue.

An extensively described axis of communication between neurons and microglia is fractalkine-CX3CL1/CX3CR1. This signaling pathway is very important in the maintenance of microglia under a vigilant phenotype. Our results showed diminished mRNA expression of CX3CR1 but increased gene expression of its ligand after demyelination, suggesting a reduced response of microglia to CX3CL1. Cardona and collaborators showed that CX3CR1 deficient microglia overexpressed IL-1β and displayed neurotoxic activity indicating that this axis might be compromised during a demyelinated insult and that microglia can acquire an activated phenotype (Cardona et al., 2006). Furthermore, CX3CR1 knockout mice showed increased toxicity in response to LPS treatment and augmented neurodegeneration (Cardona et al., 2006). Although it has already been reported an augmented expression of CX3CR1 in inflammatory lesions of MS patients (Wollberg et al., 2014), our results suggest that in our *ex vivo* demyelination model the lack of fractalkine receptor may be involved in the activation of microglia into a pro-inflammatory phenotype releasing several inflammatory mediators and promoting neurodegeneration. We also verified that with S100B neutralization, the CX3CR1 expression levels returned to control values, possibly as an attempt to restore this pathway, justifying why S100B protein, at physiological levels, was reported to prevent the activation of microglial cells (Zhang et al., 2011b). Regarding to fractalkine-CX3CL1, our results revealed an overexpression of this gene expression levels after LPC treatment and a slight increase upon co-incubation with anti-S100B antibody. Many neurodegenerative and inflammatory disorders that are associated with increased microglial activation, including MS, show disruption of the fractalkine/CX3CR1 crosstalk. Transgenic mice that develop EAE spontaneously and mice actively immunized with proteolytic peptide display an augmented fractalkine in brain microglia (Sheridan and Murphy, 2013). Therefore, a CNS injury, mimicked into our model by LPC

treatment, may be a trigger to increase CX3CL1 expression which may either contribute to disease progression or be a mechanism by which other cells attempt to stop microglia high reactivity shifting them to a quiescent/vigilant phenotype.

IgG co-incubation appears to have a similar effect of S100B neutralization in some cases, which might indicate that an introduction of an IgG to the incubation medium may have an effect by itself which may mask the intended neutralization of S100B. Therefore, it would be interesting to investigate the role of additional S100B modulators that have been already demonstrated to play a role in preventing S100B-related damage in cancer and experimental traumatic brain injury, in our *ex* vivo model of demyelination (Capoccia et al., 2015; Kabadi et al., 2015; Zimmer et al., 2013).

Concluding Remarks

With this thesis we demonstrated, using an *ex* vivo COSC model, that LPC effectively induced demyelination through reduction of myelin protein expression and confirmed that the anti-S100B treatment partially prevent this demyelination. Moreover, we showed that the demyelinated insult induced an inflammatory environment resulting from the release of inflammatory molecules by activated microglia. On the other hand, the blockade of S100B seems to prevent the expression of pro-inflammatory mediators and promote the shift microglia to a damage repair phenotype with the increase of phagocytic ability (Figure IV. 1), although M2-like markers were not induced. Overall, our results strongly suggest that inhibition of excessive S100B upon demyelination prevents both demyelination and the exacerbation of the inflammatory milieu, possibly also affecting microglia activation and the recruitment of other immune cell to CNS parenchyma. Further studies on *in vivo* MS models will better clarify whether targeting of S100B can be a good therapeutic strategy to reduce damage and increase recovery in MS.

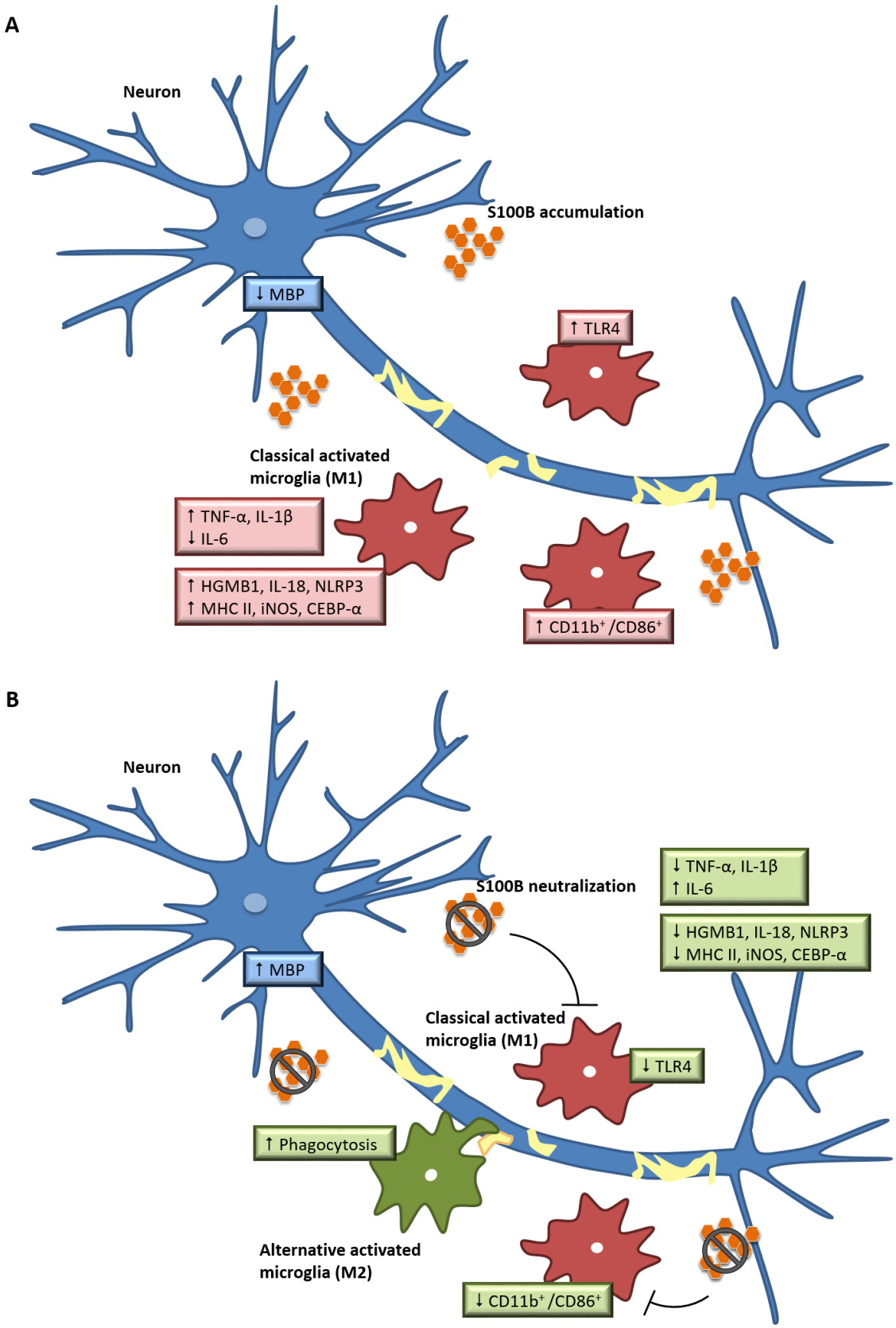

Figure IV. 1. Schematic representation of main findings. (A) In demyelinated conditions there is an upregulation of pro-inflammatory cytokines. **(B)** Blocking S100B showed a reduction of pro-inflammatory environment and an increased phagocytosis.

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